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Roles for N-Terminally Acylated Sequences from Signal Transducing Proteins in Differential Subcellular Localization

by

James B. McCabe ©

A thesis submitted to the Faculty of Graduate Studies and Research in Partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Cell Biology

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To my friends, I am sorry, for this has taken me away for years.
To my lipid modification scientific colleagues, how wonderful
it is that you picked the best field to work in!
To God, for giving me the strength to perservere,
and the ability to achieve all that I have achieved

For my mother, and parents-in-law, for believing in me
For Morganne, Landon, and Preston, who enrich me every day
And most of all, for my wife Tara, for having the patience to endure this
consuming labour of love, and for loving me for who I am

The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say.

J.R.R. Tolkien

ABSTRACT

Numerous intracellular proteins contain different lipid modifications (lipidation) on amino acid residues located throughout their primary structure. Specific membrane association of these proteins is often essential for their functional activity. However, the significance of different modifying lipids and the characterization of membrane binding signals which appear to regulate the destination of so modified proteins are only partially understood. To limit the scope of the study, the fatty acylated N-terminal sequences from the Src family of protein tyrosine kinases, G protein alpha subunits and GAP-43 were utilized in this project. These proteins are N-terminally modified by myristic and/or palmitic fatty acids, and some also contain membrane associating polybasic regions. We sought to determine whether the variably acylated N-terminal sequences could direct differential membrane association of these proteins. We appended wild-type and acylation-mutant forms of the N-terminal 11-16 amino acids from each protein to the N-terminus of green fluorescent protein (GFP). Variably acylated GFP chimeras differentially associated with endosomes, endoplasmic reticulum, Golgi apparatus, and plasma membrane in an acylation-dependent fashion. Our results suggest that acylation of an otherwise cytosolic reporter protein is a requirement for that protein to associate with membranes of a specific composition. To further investigate this, the subcellular distributions of cholesterol and sphingolipids, two lipid entities enriched in partially characterized membrane subdomains termed lipid rafts, were compared to that of fatty acylated GFPs. Fatty acylated GFPs were found to colocalize with cholesterol and sphingolipids, but did not fractionate into detergent-resistant lipid rafts, as did known lipid raft markers. We conclude that N-terminal

acylation, in combination with polybasic regions, retains or enriches proteins, in a detergent-soluble fashion, in cholesterol-, sphingolipid-rich membrane environments, notably the plasma membrane and endocytic compartments. This retention may act to orient protein-protein interaction modules to ultimately determine the final destination of the lipid-modified protein. Our findings represent an alternative interpretation as to the functional role of N-terminal lipid modifications, as the field has been dominated by the belief that the lipid modifications alone govern the enrichment of fatty acylated proteins into specific detergent-resistant membrane subdomains.

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LIST OF ABBREVIATIONS

β_2-AR	β_2-adrenergic receptor
βARK	β-adrenergic receptor kinase
^{125}I-IC16	16-[^{125}I] iodohexadecanoic acid
AAC/ANT	mitochondrial ATP/ADP carrier/adenine nucleotide transporter
Ab	antibody
ACBP	acyl-CoA binding protein
ACS	acyl-CoA synthetase
ADP	adenosine 5’-disphosphate
AKAP	A-kinase (PKA) anchoring protein
APT	acyl protein thioesterase
ARF/Arf	ADP-ribosylation factor
ATP	adenosine 5’-triphosphate
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-5-indacene fluorophore
BFA	Brefeldin A
BSA	bovine serum albumin
CAAX	C-terminal prenylation signal sequence
CaM	calmodulin
cAMP	3’,5’-cyclic adenosine monophosphate
cDNA	complementary DNA
Chol:MβCD	cholesterol:methyl β-cyclodextrin complex
Ci	Curie
CI-MPR	cation-independent mannose 6-phosphate receptor
CMV	cytomegalovirus
CoA	coenzyme A
COS-7	CV-1 derived SV40-transformed African green monkey kidney cell line
CPS 1	carbamoyl phosphate synthetase 1
DAF	decay accelerating factor
dATP	deoxyadenosine 5′-triphosphate
dCTP	deoxycytidine 5′-triphosphate
DEAE	diethylaminoethyl
dGTP	deoxyguanosine 5′-triphosphate
DIGs	detergent-insoluble glycosphingolipid enriched membranes
DiI-LDL	1, 1′-hexadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate-low density lipoprotein fluorophore complex
DIM	detergent insoluble membrane
DMEM	Dulbecco’s Modified Eagle’s Medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
dNTP	deoxyribonucleotide 5′-triphosphate
DOPC	dioleoylphosphatidylcholine
DPPC	dipalmitoylphosphatidylcholine
DRM	detergent-resistant membrane
DTT	dithiothreitol

dTTP	deoxythymidine 5′-triphosphate
ECL	enhanced chemiluminescence
ecNOS	endothelial cell nitric oxide synthase
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosome antigen-1
EGFP	enhanced GFP
ER	endoplasmic reticulum
ERC	endosomal recycling compartment
ERGIC	ER-Golgi intermediate compartment
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FITC-CTX	FITC-conjugated cholera toxin B subunit
FRAP	fluorescence recovery after photobleaching
GAD	glutamic acid decarboxylase
GAP-43/NM	growth cone associated protein of 50 kDa/neuromodulin
GDP	guanosine 5′-diphosphate
GEM	glycolipid-enriched membranes
GFP	green fluorescent protein
GM$_1$	sphingolipid ganglioside GM$_1$
GPCR	G protein-coupled receptor
GPI	glycosylphosphatidyl inositol lipid anchor
GTP	deoxyguanosine 5′-triphosphate
HA	hemagglutinin protein
HASP	hydrophilic acylated surface protein
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IC16	iodopalmitate analogue
IgG	immunoglobulin G
IL	interleukin
IP	immunoprecipitation
LAMP	lysosome associated membrane protein
LAT	linker of activated T cells
l_d	liquid-disordered membrane phase
l_o	liquid-ordered membrane phase
LPDS	lipoprotein-deficient fetal bovine serum
MβCD	methyl β-cyclodextrin
MAP	methionyl aminopeptidase
MARCKS	myristoylated alanine-rich C-kinase substrate
MCS	multiple cloning site
MES	2-(N-morpholino)ethanesulfonic acid
MMSDH	methylmalonyl semialdehyde dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAT	*N*-acetyltransferase
NDS	normal donkey serum

NLS	nuclear localization sequence
NMT	*N*-myristoyl transferase
NP-40	Nonidet P-40
NRTK	nonreceptor tyrosine kinase
NZ	nocodazole
OG	*n*-octyl β-D-glucopyranoside (*n*-octyl glucoside)
P100	particulate fraction resulting from 100 000 x *g* centrifugation
PAGE	polyacrylamide gel electrophoresis
PAT	protein *S*-/palmitoyl-acyltransferase
PBS	phosphate buffered saline
PBST	phosphate buffered saline + 0.1% Tween-20
PCR	polymerase chain reaction
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP$_2$	phosphatidylinositol 4,5-*bis*phosphate
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PKA	protein kinase A/cAMP-dependent protein kinase
PKC	protein kinase C
PLAP	placental alkaline phosphatase
PLP	brain myelin proteolipid protein
PMSF	phenylmethylsulfonylfluoride
PPT	palmitoyl protein thioesterase
PS	phosphatidylserine
PSD-95	postsynaptic density protein of 95 kDa
PTK	protein tyrosine kinase
PVDF	polyvinylidenelfluoride
RGS	regulator of G protein signaling protein
RPE	retinal pigment epithelium
S100	soluble fraction resulting from 100 000 x *g* centrifugation
SDS	sodium dodecyl sulfate
SFV	Semliki Forest virus
SH2	Src homology 2 protein-protein interaction domain
SH3	Src homology 3 protein-protein interaction domain
SOE	splicing by overlap extension
SV40	simian virus 40
TE	Tris-EDTA
Tf	transferrin
T$_m$	acyl chain melting temperature (fluid-gel transition point)
TMD	transmembrane domain
TNF	tumour necrosis factor
TR	Texas Red
Tris	Tris(hydroxymethyl)aminomethane
TX-100	Triton X-100
VSV G	vesicular stomatitis virus protein G

CHAPTER ONE

INTRODUCTION

1.1 COVALENT LIPID MODIFICATION OF PROTEINS - OVERVIEW

The synthesis and degradation pathways for individual proteins often involve numerous co- and post-translational modifying steps.

> *Co- and post-translational events often have the same importance to functional protein production as the transcriptional and translational events that initiate their synthesis* (Rucker and McGee, 1993).

At the levels of co- and post-translation, specific amino acid modifications are often introduced to signal vectoral transport or compartmentalization to a given cellular location, regulate enzyme or protein activity or longevity, add or form a prosthetic group, and create metal ion binding sites. These modifications extend the range of chemical properties of amino acids well beyond those of the 20 amino acids used for protein synthesis. Over 400 amino acid derivatives have now been identified as novel products of amino acid modifications. Many of these involve lipid modifications, also known as lipidation. **Table 1.1** illustrates a contemporary summary of lipid modifications.

The earliest report describing the phenomenon of protein lipidation was Folch and Lees' description of brain myelin lipidation in 1951 (Folch and Lees, 1951). The introduction of protein lipidation as a modern field of study began some thirty years later with the discovery of viral glycoprotein lipidation in eukaryotic cells (Schmidt *et al.*, 1979; Schmidt and Schlesinger, 1979). In the past two decades, the study of protein lipidation has identified several hundred diverse lipid-modified proteins that vary in function and subcellular localization (**Figure 1.1**). These proteins are covalently modified by a variety of lipids and lipid combinations. The lipid modifications appear to function by stabilizing tertiary protein structure, modulating protein-protein interactions, regulating enzymatic activity, or imparting hydrophobicity, which may facilitate their association with various cellular membranes. In turn, membrane association allows specific protein-protein interactions to occur that are essential for a number of distinct cellular processes.

The covalent lipid modification of proteins is traditionally divided into three categories: acylation, which encompasses myristoylation and palmitoylation, prenylation; which encompasses farnesylation and geranylgeranylation; and glypiation, which is the

Table 1.1: Lipid Modifications involving N- and C-terminal and internal amino acids

Process	Target Residues	Modified Proteins	Substrates, Cofactors	Possible Functions	References
N-terminal acylation (amide-linked)	**Gly** Cys	**Many Gα subunits, NRTKs** Also Ca^{2+}-binding, viral proteins Myelin basic protein $G\alpha_s$ Sonic Hedgehog and family	**Myristoyl-CoA** (C12:0, C14:1, C14:2)* C2-10 fatty acyl-CoAs Palmitoyl-CoA Palmitoyl-CoA	Compartmentalization Protection from proteolysis Protein-protein interaction *Increase Signaling Rate	(Dizhoor *et al.*, 1992; Farazi *et al.*, 2001; Kleuss, 2000; Moscarello *et al.*, 1992; Neubert *et al.*, 1992; Pepinsky *et al.*, 1998)
Internal acylation (thioester) (amide) (oxyester) (Schiff base)	**Cys** Lys Ser, Thr Lys	**G_α subunits, NRTKs, GAP-43** Also GPCRs, viral proteins MMSDH, CPS-1 Cytokeratins, PKA TNF-α, IL-1α precursors ghrelin rhodopsin RPE retinal GPCR	**Palmitoyl-CoA** (C14:0, C18:0/1, C20:4, C22:5) C8-C18 fatty acyl-CoAs All-*trans*-retinoic acid Myristoyl-CoA Octanoyl-, Decanoyl-CoA 11-*cis* retinal all-*trans* retinal	Compartmentalization, Protein-protein interaction Active site regulation unknown Compartmentalization Receptor Binding Prosthetic group	(Berthiaume *et al.*, 1994; Buczylko *et al.*, 1996; Corvi *et al.*, 2001; Dunphy and Linder, 1998; Hao and Fong, 1999; Kaiya *et al.*, 2001; Kojima *et al.*, 1999; Laposata and Muszbek, 1996; Resh, 1999; Stevenson *et al.*, 1993; Takahashi *et al.*, 1991)
Internal phospho-inositidation	Ser	Myelin basic protein	PIP, PIP_2	Membrane binding, signaling?	(Chang *et al.*, 1986; Yang *et al.*, 1986)
C-terminal phosphatidylethanol aminoylation	Gly	Apg8/Aut7, essential for yeast autophagy	phosphatidylethanolamine	Compartmentalization, Protein-protein interaction	(Ichimura *et al.*, 2000)
C-terminal glypiation (amide)	Ala, Asn, Asp, Cys, Gly or Ser	acetylcholinesterase, PLAP, CD59, Thy-1, folate receptor	GPI (glycosylphosphatidyl-inositol)	Compartmentalization, Protein-protein interaction	(Micanovic *et al.*, 1990)
C-terminal cholesteroylation (oxyester)	Gly	Sonic hedgehog and homologs	Cholesterol	Compartmentalization, Protein-protein interaction	(Porter *et al.*, 1996)
C-terminal prenylation (thioether)	Cys (in CAAX, CC or CXC motifs)	Ras superfamily, Rab proteins, Rho, G protein γ subunits	Farnesyl (C15) geranylgeranyl (C20)	Compartmentalization, Protein-protein interaction	(Prior and Hancock, 2001) (Zhang and Casey, 1996)

Modifications in **bold** emphasize the modifications and target proteins investigated in this project

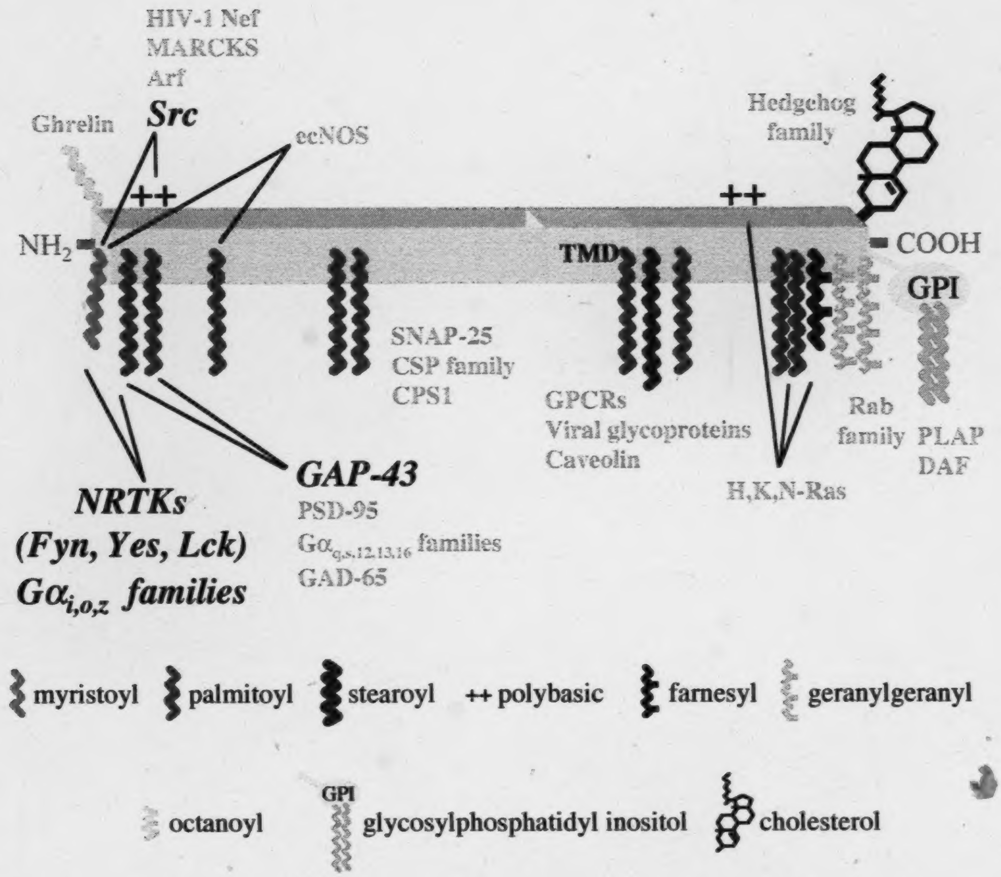
HIV-1 Nef
MARCKS
Arf
Src
Ghrelin
ecNOS
Hedgehog family
++
++
NH₂
TMD
COOH
GPI
SNAP-25
CSP family
CPS1
GPCRs
Viral glycoproteins
Caveolin
Rab family
PLAP
DAF
H,K,N-Ras
GAP-43
PSD-95
Gα q,s,12,13,16 families
GAD-65
NRTKs
(Fyn, Yes, Lck)
Gα i,o,z families
myristoyl
palmitoyl
stearoyl
++ polybasic
farnesyl
geranylgeranyl
octanoyl
GPI
glycosylphosphatidyl inositol
cholesterol

Figure 1.1. Diversity of lipid modification in cellular proteins. Locations of selected lipid modifications in the primary sequence of selected proteins. Symbols below the figure indicate the particular attached lipid moiety. The green rectangle represents the polypeptide backbone of lipid-modified proteins. TMD: transmembrane domain. Proteins modified at their N-termini by combinations of myristoylation, palmitoylation, or polybasic regions (highlighted in bold italics) were chosen to be the focus of this project.

modification of cell surface proteins with the glycosyl phosphatidylinositol (GPI) glycolipid (**Table 1.1**). For classification purposes, a fourth category may be described as "novel lipidation" and includes covalent modification of specific proteins with retinoic acid (Takahashi and Breitman, 1989; Breitman and Takahashi, 1996; Myhre *et al.*, 1996), cholesterol (Porter *et al.*, 1996), phosphatidylethanolamine (Ichimura *et al.*, 2000), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-*bis*phosphate (PIP_2) (Chang *et al.*, 1986; Yang *et al.*, 1986), and *n*-octanoyl or *n*-decanoyl groups (Kojima *et al.*, 1999; Kaiya *et al.*, 2001).

1.2 N-TERMINAL PROTEIN ACYLATION

Protein acylation most commonly refers to the modification of proteins by myristate (C14:0), a saturated 14-carbon fatty acid, and/or palmitate (C16:0), a saturated 16-carbon fatty acid (**Figure 1.2**, and **Table 1.1**). Proteins may also be modified by laurate (C12:0), myristoleate {(C14:1*n*-9) or (*cis*-Δ5)-tetradecaenoyl}, (*cis,cis*-Δ5,Δ8)-tetradecadienoyl (C14:2*n*-6) (Kokame *et al.*, 1992), palmitoleate (C16:1*n*-9) (Casey *et al.*, 1994), stearate (C18:0) (Veit *et al.*, 1996; Van Cott *et al.*, 1997), oleate (C18:1*n*-9) (Bizzozero *et al.*, 1986; Muszbek *et al.*, 1999) and *cis*-5,8,11,14,17-eicosapentaenoate and arachidonate {C20:4 (5,8,11,14)} (Hallak *et al.*, 1994; Laposata and Muszbek, 1996). The prevalence of intracellular acyl-CoA species does not appear to reflect the preference for these fatty acids in all cases, as some, including myristate which constitutes 4-7% of total cellular acyl-CoA pools, are rare fatty acids (Buss *et al.*, 1984; DeMar and Anderson, 1997). The vast majority of acylated proteins are modified by two lipid species, myristate and palmitate, and their covalent linkage to the N-terminal region of signaling proteins will remain the focus for the remainder of this study.

1.3 MYRISTOYLATION

1.3.1 Historical Perspectives

Protein myristoylation was first described in 1982 for calcineurin B and the catalytic subunit of protein kinase A (PKA) after the identification of an N-terminal blocking group as myristate (Aitken *et al.*, 1982; Carr *et al.*, 1982). Around the same

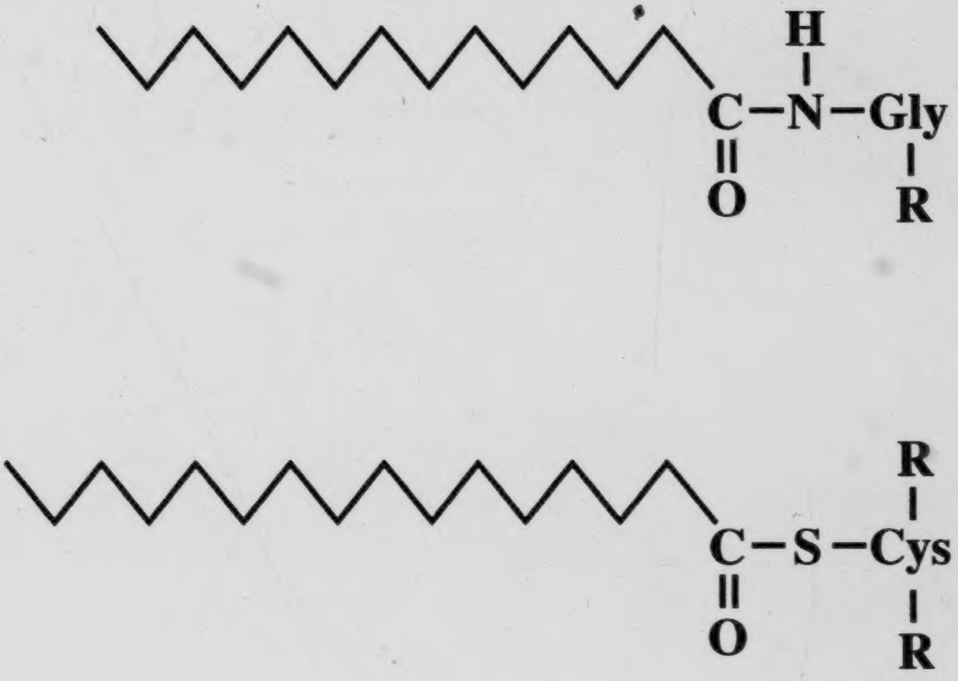

Figure 1.2. Structures of lipid modifications found at or near the N-termini of cellular proteins. R denotes continuation of the polypeptide backbone. Upper structure depicts N-terminal amide-linked myristoylation, and the lower structure depicts thioester-linked palmitoylation.

time, investigators discovered the myristoylation of murine and avian retroviral structural and signaling proteins, such as p15gag and v-src (Henderson *et al.*, 1982; Sefton *et al.*, 1982; Henderson *et al.*, 1983; Schultz and Oroszlan, 1983; Buss *et al.*, 1984; Cross *et al.*, 1984; Schultz and Oroszlan, 1984; Buss and Sefton, 1985; Garber *et al.*, 1985). Additional studies suggested a critical role for myristoylation in virus assembly, cell infectivity, and virion stability (Rein *et al.*, 1986; Chow *et al.*, 1987; Dorval *et al.*, 1989; Marc *et al.*, 1989; Schmidt *et al.*, 1989; Schultz and Rein, 1989; Lee and Chow, 1992). These early studies revealed the cotranslational nature and metabolic stability of myristoylation; furthermore, these early studies suggested that myristoylation was not sufficient to convert all soluble proteins to membrane-bound forms (Buss *et al.*, 1984; Olson *et al.*, 1985).

The transforming capability and membrane binding of the avian Rous sarcoma virus gene product (v-src) was abolished when the protein could not be myristoylated by deleting the N-terminal 80 amino acids, despite retaining tyrosine kinase activity (Cross *et al.*, 1984; Schultz *et al.*, 1985). However, a myristoylated v-src internal deletion (kinase inactive) mutant was not membrane bound and was transformation defective. These "loss-of-function" results suggested that membrane binding of functional protein, not myristoylation, was essential for transformation (Garber *et al.*, 1985). Kamps *et al.* suggested myristoylation of v-src was essential for membrane association and transforming activity, when they mutated selectively the N-terminal glycine to alanine, creating a kinase active nonmyristoylated mutant (Kamps *et al.*, 1985). A nonmyristoylated mutant of the viral oncogene v-abl was found to transform hematopoietic but not fibroblast cell lines, suggesting that the relationship between transformation, myristoylation, and membrane association was more complex than initially believed (Daley *et al.*, 1992). To further illustrate this, v-src was later found to contain independent domains that target it to distinctive subcellular locations (Kaplan *et al.*, 1990).

The creation of acylated chimeric proteins to assess "gain-of-function" was first introduced by Pellman *et al.*, who created fusion proteins containing the N-terminal 14 amino acids of v-src fused to either the nonacylated retroviral fgs gene product or to the chimpanzee α-globin gene product (Pellman *et al.*, 1985). This important study, and others, showed that plasma membrane (PM) association could be achieved with the addition of these small N-terminal acylation sequences (Lacal *et al.*, 1988).

A proteinaceous receptor for myristoylated proteins was first proposed by Resh and coworkers (Resh, 1988). Work with myristoylated peptides modeled after the N-terminus of Src showed high-affinity binding to a membrane protein, later identified as the inner mitochondrial membrane protein ADP/ATP carrier (AAC) (Goddard *et al.*, 1989; Resh, 1989; Resh and Ling, 1990; Sigal and Resh, 1993). Retrospectively, this result emphasized that caution is required when comparing binding affinities and specificity of myristoylated peptides versus full-length proteins (Silverman *et al.*, 1992). To date, no receptor for myristoylated proteins has been identified.

Many of the Src and Abl families of nonreceptor tyrosine kinase (NRTK) proteins are myristoylated (Src, Yes, Lck, Fyn, Lyn, Hck, Fgr, Yrk, Blk, Abl, and Arg) (Kypta *et al.*, 1988; Wang and Hanafusa, 1988; Peters *et al.*, 1990; Nadler *et al.*, 1993; Shenoy-Scaria *et al.*, 1993; Silverman *et al.*, 1993; Wang and Kruh, 1996). In addition, it was discovered that GTP-binding proteins, including G protein α subunits and ADP-ribosylation factor (ARF) were covalently modified by myristate, utilized for membrane association and increased binding with the $\beta\gamma$ subunit in some cases (Buss *et al.*, 1987; Kahn *et al.*, 1988; Mumby *et al.*, 1990; Linder *et al.*, 1991; Denker *et al.*, 1992; Haun *et al.*, 1993). Retinal photoreceptors were found in 1992 to be the only cell type to heterogeneously acylate proteins (with shorter and unsaturated fatty acids), disproving the belief that proteins *in vivo* are exclusively modified by myristate at the N-terminus (Dizhoor *et al.*, 1992; Kokame *et al.*, 1992; Neubert *et al.*, 1992; Johnson *et al.*, 1994b).

Studies in the 1990s by Silvius, McLaughlin, Thompson *et al.* shed light on the biophysical aspects of protein *N*-myristoylation and membrane association, and made significant contributions in understanding why the cell selects such a rare fatty acid to be attached at the N-terminus (Shahinian and Silvius, 1995; Murray *et al.*, 1997; Pool and Thompson, 1998). The combination of an electrostatic membrane binding motif with myristoylation was first hinted at when a lysine at position 7 of the N-terminal v-src sequence was found to improve v-src acylation, and was considered part of the consensus motif later found in a number of NRTKs (Kaplan *et al.*, 1988; Silverman and Resh, 1992; Silverman *et al.*, 1993). Complementary studies on Ras lipidation serendipitously revealed multiple lipid modifications at the C-terminus of Ras isoforms H-Ras and N-Ras, but not K-Ras. K-Ras was found to have an extensive polybasic domain adjacent to the farnesylated C-terminal cysteine. Studies with Ras demonstrated that either a polybasic domain or palmitoylation was required for stable membrane association of these Ras isoforms (Hancock *et al.*, 1990). A synergistic effect between myristoylation and a polybasic domain in membrane binding was demonstrated to be sufficiently strong enough to anchor proteins to acidic phospholipid-containing membranes (Buser *et al.*, 1994; Cadwallader *et al.*, 1994; Sigal *et al.*, 1994; Zhou *et al.*, 1994; Zhou and Resh, 1996). The regulation of membrane binding of this electrostatic domain by introduction of charge-neutralizing phosphate groups via phosphorylation was shown elegantly in work on the myristoylated alanine-rich C-kinase substrate (MARCKS) protein, and the concept of "myristoyl switches" was first introduced (Taniguchi and Manenti, 1993; McLaughlin and Aderem, 1995; Murray *et al.*, 1997; Murray *et al.*, 1998). Concurrent investigations with myristoylated G proteins and NRTKs implicated the acylation of these proteins in targeting to caveolae and detergent-resistant membranes (DRMs)/lipid rafts, since loss of myristoylation led to loss of membrane localization and subsequent loss of lipid raft localization (Sargiacomo *et al.*, 1993; Li *et al.*, 1995; Shaul *et al.*, 1996; Song *et al.*, 1997a).

Two notable additions to our understanding of myristoylation have surfaced. Firstly, myristoylation of the ε-amino group on internal lysine residues was reported for the 31 kDa interleukin (IL) IL-1α propiece and for the tumour necrosis factor (TNF) α precursor (Stevenson *et al.*, 1992; Stevenson *et al.*, 1993). Apparently to promote membrane association, the modified lysines in the IL-1α propiece are within a nuclear localization sequence (NLS). Lysine myristoylation may represent a means for regulating the activity of the NLS, through loss of positive charge and abrogation of NLS function. Transport of the IL-1α propiece into the nucleus is required for it to modulate endothelial cell function (Maier *et al.*, 1994). An uncharacterized lysyl ε-amino *N*-myristoyltransferase activity may facilitate its specific membrane targeting (Stevenson *et al.*, 1997). Secondly, although long believed to be only co-translational, the recent discovery of post-translational post-proteolytic myristoylation has been demonstrated in the pro-apoptotic molecule BID. After BID cleavage by caspase 8, an N-terminal NMT myristoylation consensus sequence is exposed and acylated post-proteolytically to target the mitochondrial membrane and initiate caspase-mediated apoptosis (Zha *et al.*, 2000).

1.3.2 Enzymology of N-Myristoylation

The enzymology of *N*-myristoylation has been extensively characterized (Johnson *et al.*, 1994a; Farazi *et al.*, 2001). N-myristoyl transferase (NMT; EC 2.3.1.97) is a 48-68-kDa monomeric enzyme that catalyzes transfer of myristate from myristoyl-CoA to suitable peptide and protein substrates. NMT was purified and characterized in 1987 (Towler *et al.*, 1987). To date, 19 NMT genes from fungal, protozoal, nematode, plant, insect and mammalian sources have been identified (Farazi *et al.*, 2001). Mammalian species each possess two NMTs (type I and type II), divergent at their N-termini. Type I and II NMTs contain an extended N-terminal sequence (as compared to the yeast gene) for ribosome binding. Alternative splicing of the NMT I mRNA leads to four distinct protein isoforms (full-length and truncated forms). This would allow for both ribosomal co-translational and cytosolic post-translational myristoylation to occur (Giang and Cravatt, 1998; Farazi *et al.*, 2001). Studies of NMTs from the yeast *Saccharomyces cerevisiae* and *Homo sapiens* reveal that the catalytic cycle exhibits the following sequential ordered Bi Bi reaction mechanism: (1) myristoyl-CoA binds to NMT; (2) the peptide substrate binds to NMT; (3) myristate is transferred to the N-terminal glycine of the peptide; (4) CoA is released from the enzyme; (5) myristoyl-peptide is released (Rudnick *et al.*, 1991; Rocque *et al.*, 1993).

N-myristoylation is primarily a co-translational process that occurs while the nascent polypeptide chain (< 100 amino acids) is still attached to the ribosome (Wilcox *et al.*, 1987; Deichaite *et al.*, 1988). It occurs at the N-terminus of proteins beginning with certain consensus sequences, typically $MGX_3X_4X_5Ser/CysX_7X_8X_9$, where X represents almost any amino acid. However, neutral residues are preferred in positions X_{3-5}, and basic residues in X_{7-9} (**Table 1.2**). Higher eukaryotic organisms appear to have less stringent requirements for consensus (Rocque *et al.*, 1993; Utsumi *et al.*, 2001). Unequivocally needed is an N-terminal glycine residue; substitution of glycine with any other amino acid abrogates myristoylation (Rudnick *et al.*, 1990). Not all proteins with an

Table 1.2: Consensus Sequence in N-Myristoyl Proteins

N-Terminal Amino Acid Position of Modified Protein									Comments
1	2	3	4	5	6	7	8	9	Note: positions 8 and 9 make very few contacts with N-myristoyl transferase (NMT)
(Met)	**Gly**	X	X	X	X	X	X	X	Basic Requirement. (Met): cleaved by MAP and Gly, exposed for myristoylation by NMT
		uncharged (Cys, Ala, **Leu**)	**neutral**>basic> acidic		**Ser**	Thr/ Lys/ Leu	**basic**>neutral/ acidic		Preferred by yeast Nmtp (more strict) **Bold** indicates most preferred by Nmtp
		Ser, Thr, Val, Asn, Ile, Gln, His, Lys, Arg, Asp allowed	bulkier/more polar allowed		Thr, Cys, Ala, Gly, Val, Glu		Acidic OK		Allowances by hNMT (more flexible)
(Met)	Gly, Ala, Ser, Cys, Thr, Pro, Val								**Preferences of MAP** Gyration Radius of amino acid 2 side chain <1.29 Å : Met removal >1.29 Å : Met retention
Met	Asp, Asn, Leu, Ile, Gln, Glu, His, Met, Phe, Lys, Tyr, Trp, Arg								
lipid									
Myr	Gly	Ala, Ser, Cys, Thr, Val, Asn, Leu, Ile, Gln, His (Lys/Arg/ Asp *in vivo*)	Ser	Ser	**Ser**	Arg	Thr	Pro	**MGXXXS/A-TNF chimeras** Gyration radius of amino acid 3 side chain <1.80 Å: myristoylated *in vitro* >1.80 Å/steric/acidic: not myristoylated
Ac	Gly	Asp and Glu (acidic)	Ser	Ser	**Ser**	Arg	Thr	Pro	Downstream sequences must play a role in proteins with a Lys/Arg/Asp at position 3
None	Gly	Phe, Lys, Tyr, Trp, Arg, Pro	Ser	Ser	**Ser**	Arg	Thr	Pro	
Myr/Ac	Gly	Gly and Met	Ser	Ser	**Ser**	Arg	Thr	Pro	**Position 3 affects co-translational acylation by NMT or NAT**
Myr	Gly	Asn and Gln	Ser	Ser	**Ala**	Arg	Thr	Pro	**Position 6 affects specificity at position 3 for NMT (fewer substrates) and NAT (more substrates)**
Ac	Gly	Ser, Thr, Asp, Glu, Gly, Met	Ser	Ser	**Ala**	Arg	Thr	Pro	

Myr: myristoylated; ***Ac***: acetylated; **hNMT**: human NMT; **NMTp**: yeast NMT; **MAP**: methionyl aminopeptidase; **NAT**: *N*-acetyl transferase

(Arfin and Bradshaw, 1988; Kishore *et al.*, 1991; Kishore *et al.*, 1993; Rocque *et al.*, 1993; Utsumi *et al.*, 2001)

N-terminal glycine are *N*-myristoylated; the ability to be recognized by NMT versus other competing enzymes (such as *N*-acetyltransferase, NAT) is also important (Bradshaw *et al.*, 1998; Utsumi *et al.*, 2001). Methionyl aminopeptidases (MAPs) involved in the co-translational removal of the initiator Met are defined by a highly conserved substrate specificity, which is dictated by the residue adjacent to the Met residue (Tsunasawa *et al.*, 1985; Moerschell *et al.*, 1990). The seven amino acids that have the smallest radii of gyration are substrates for MAPs, while those with the 13 largest side chains are not. Residues downstream of the specificity-determining residue have little impact on the reaction. NATs that catalyze co-translational protein *N*-acetylation also have a restricted number of substrates (Huang *et al.*, 1987; Boissel *et al.*, 1988; Bradshaw *et al.*, 1998; Arnold *et al.*, 1999; Polevoda *et al.*, 1999). Studies both in yeast and in higher eukaryotic cells showed that the principal substrates of NATs are proteins that have N-terminal Gly, Ala, Ser, or Thr residues (GAST substrates), or Met residue (M substrates) (Lee *et al.*, 1988; Mullen *et al.*, 1989; Yamada and Bradshaw, 1991). In the case of M substrates, *N*-acetylation only occurs if the adjacent residue is an Asp, Glu or Asn residue. The amino acid requirements for the adjacent residue of the GAST substrates have not been defined as yet. Unlike the MAPs, this specificity is clearly affected by downstream residues, making the rules that guide acetylation less clear **(Table 1.2)**.

In addition, NMT is highly selective for myristoyl-CoA, and shorter or longer fatty acyl-CoAs are not transferred efficiently. It is interesting to note that palmitoyl-CoA binds to NMT with approximately the same affinity as myristoyl-CoA, although palmitate is not transferred by NMT to proteins (Kishore *et al.*, 1993). Since the concentration of palmitoyl-CoA is 5- to 20-fold higher than myristoyl-CoA in most cells, a mechanism must exist to prevent palmitoyl-CoA from functioning as a competitive inhibitor of NMT *in vivo*. It has been proposed that due to its higher hydrophobicity, most of the palmitoyl-CoA is sequestered in cell membranes or by the housekeeping protein acyl-CoA binding protein (ACBP), and therefore may not be freely accessible to cytoplasmic/ribosomal NMT (Marc *et al.*, 1989; Peitzsch and McLaughlin, 1993; Johnson *et al.*, 1994a; Faergeman and Knudsen, 1997; Knudsen *et al.*, 2000). As such, the specificity for myristate may be enhanced by the relatively weak affinity of this substrate for membranes compared to palmitoyl-CoA and other longer chain CoAs. Free acyl-CoAs partition into membranes in a chain-length and saturation-level dependent fashion. Cytoplasmic ACBPs may also play a role in delivering myristoyl-CoA to NMT or contribute to the acyl chain specificity of the enzyme (Bhatnagar and Gordon, 1997). Unfortunately, the compartmentalization of long-chain acyl-CoA esters, and the free cytosolic concentration of these species, are important unresolved issues (Faergeman and Knudsen, 1997).

Heterogeneous N-terminal acylation involving the unsaturated fatty acids C14:1 and C14:2 and C12:0 (laurate) has been seen exclusively in retinal photoreceptor cells, despite having fatty acyl-CoA pools nearly identical to other tissues (Dizhoor *et al.*, 1992; Neubert *et al.*, 1992; DeMar and Anderson, 1997). This substrate flexibility is due in part to the NMT's acyl-CoA binding pocket, which binds the acyl chain in a bent conformation with bends located in the vicinity of C5 and C8 of the acyl chain (Kishore *et al.*, 1991; Kishore *et al.*, 1993). This makes it well suited to utilize C14:2 and C14:1 fatty acids, which have bends due to double bonds at these locations. Additional unidentified factors must be at play, since heterogeneous acylation is not seen in other tissues studied to date and NMT is ubiquitously expressed. X-ray crystallographic studies demonstrate a flexibility in the acyl-CoA binding pocket to accommodate these additional rare acyl substrates (Bhatnagar *et al.*, 1998; Bhatnagar *et al.*, 1999). Such alternate unsaturated fatty acids can be incorporated into acylated proteins by supplementing the growth media of cultured cells with them (Liang *et al.*, 2001).

Myristate is covalently bound to the protein by an amide bond, and the modification is essentially irreversible, with a half-life equivalent to the half-life of the modified protein (Buss *et al.*, 1984; Wolven *et al.*, 1997). An exception to this permanency is a demyristoylation mechanism involving the MARCKS protein. A phosphorylated demyristoylated protein is formed when incubated with a brain cytoplasmic synaptosome fraction (Manenti *et al.*, 1994, 1995). This deacylated product can be remyristoylated in the presence of myristoyl-CoA and NMT, demonstrating that cycles of myristoylation/demyristoylation of MARCKS can be achieved *in vitro*. Braun *et al.* also demonstrated a myristoylation-dependent N-terminal cleavage between residues Lys6 and Thr7 of MARCKS exclusively by macrophage extracts (Braun *et al.*, 2000).

Thus, myristoylation is predominately the attachment of myristate (C14:0) in a co-translational manner for the apparent life of a protein. However, a few select tissues and proteins have evolved with additional regulatory mechanisms involving the identity of the fatty acid and the permanency of the modification. These alterations are likely to play a role in the function of these proteins and the signalling cascades they control in those tissues.

1.3.3 Subcellular Location of Myristoylated Proteins

Greater than one hundred and fifty myristoylated proteins have been identified (**Table 1.3**), which vary in function and subcellular localization (Resh, 1996, 1999). Myristoylated proteins include the Src family of tyrosine kinases, Gα subunits of heterotrimeric G-proteins, retroviral proteins such as HIV-1 Gag and Nef, and those involved in a variety of cellular processes, such as MARCKS, recoverin and endothelial nitric oxide synthase (ecNOS). Proteins modified by myristate may be membrane-associated, localizing to the plasma membrane (Alland *et al.*, 1994), endoplasmic reticulum (Borgese *et al.*, 1996), Golgi (Haun *et al.*, 1993; Barr *et al.*, 1997) and mitochondrial outer membranes (Borgese *et al.*, 1996; Zha *et al.*, 2000). They may also associate with the cytoskeleton (Van Etten *et al.*, 1994) and nucleus, in the case of the polyoma virus VP2 protein, HIV-1 Gag protein, and PKA catalytic subunit (Streuli and Griffin, 1987; Gallay *et al.*, 1995; Pepperkok *et al.*, 2000). Some proteins, such as calcineurin B, appear to be constitutively soluble in the cytoplasm, whereas others such as recoverin and ARF become soluble by sequestering the myristate moiety within hydrophobic pockets formed by the three-dimensional protein structure (Haun *et al.*, 1993; Ames *et al.*, 1994; Tanaka *et al.*, 1995; Goldberg, 1998).

1.3.4 Functions of Protein Myristoylation

Myristoylation of proteins serves several functions; it stabilizes tertiary protein structure and thermostability, as with the catalytic subunit of PKA (Yonemoto *et al.*, 1993; Zheng *et al.*, 1993), calcineurin B (Kennedy *et al.*, 1996), and with the poliovirus VP4 protein (Chow *et al.*, 1987). Crystal structures of these proteins provide evidence that myristate does not always protrude from the surface of a modified protein.

Table 1.3: Myristoylated Proteins

PROTEIN KINASES AND PHOSPHATASES	
Src Family Tyrosine Kinases	
Blk Fgr Fyn Hck Lck	Lyn Src Yes Yrk
Abl Tyrosine Kinases	
c-Abl type Ib	Arg B
Serine/threonine kinases, anchoring proteins	
AKAP15, 18 AK1β AMPK β-1 cGK II DAKAP200 CpCPK1 (*C. pepo L.*)	MPSK OSCPK2 PKAα, β1, γ catalytic subunit Vps15 (yeast) Yeast protein kinases
Phosphatases	
Calcineurin B PPEF (*C. elegans*) PPZ1, 2	Yeast phosphatase Yeast Psr1p/Psr2p
G PROTEINS	
Gα Subunits	
$G\alpha_{i1,2,3}$ $G\alpha_o$ $G\alpha_t$	$G\alpha_z$ Yeast Gpa1, 2
ADP-ribosylation factors	
Arf-1,2,3,4,5,6	Arl1
Miscellaneous	
Ara6 (*A. thaliana*)	RasGRP2
Ca^{2+}-BINDING/EF-HAND PROTEINS	
Aplycalcin Ca^{2+} binding protein P22 CaBP1, 2 (brain, retina) Calmyrin FCaBP (*T. cruzi*) Frequenin GCAP-1, 2 Hippocalcin	NAP-22/CAP-23 NCS-1 Neurocalcin δ Recoverin Rem-1 S-modulin SOS3 (*A. thaliana*) Visinin proteins (VILIPs)

MEMBRANE/CYTOSKELETAL STRUCTURAL PROTEINS	
Annexin XIIIa, b F52 Hisactophilin I, II Kinectin MacMARCKS	MARCKS MRP Nullo Pallidin (Band 4.2) Rapsyn
VIRAL PROTEINS	
Gag proteins	
Baboon endogenous Bovine leukemia FBR murine osteosarcoma Feline sarcoma virus (G-R) FIV Friend murine leukemia HIV-1 HTLV-1, 2	Mason-Pfizer monkey Moloney murine leukemia Moloney murine sarcoma Mouse mammary tumor Rasheed sarcoma Rauscher murine leukemia SIV Spleen necrosis
Other viral proteins	
Adenovirus 5 E16 African swine fever virus pp220, pp28, pp13 Bovine enterovirus VP4 Budgerigar fledgling disease VP2 Chlorella virus Vp260 (PBCV-1) Cowpox ATI protein Duck hepatitis B virus pre-S Encephalomyocarditis VP4 Equine herpes UL1 Foot-and-mouth VP4 Hepatitis B virus pre-SI	HIV-1,2 Nef HSV1, 2 UL11, ICP10 Murine lymphoma v-akt Poliovirus VP4 Polyomavirus VP2 Reovirus μ1 Rhinovirus 16 VP4 Rotavirus VP2, VP6 Rous sarcoma v-src SV40 VP2 Vaccinia G9R, L1R, A16L, E7R, 92 kDa ATI proteins VZV gE, Lp7
MISCELLANEOUS	
(2',5') oligoA synthetase Avr proteins (*P. syringae*) BASP1 BID (posttranslational) cPLA$_2$-γ DAPP1 ecNOS Enteropeptidase precursor FRS2	GRASP-55, -65 HASPB (*L. major*) NADH cytochrome b$_5$ reductase NADH-ubiquinone Oxidoreductase B18 OCA-B (posttranslational) P19L lens protein RP2 retinal protein Yeast Vac8p
LYSINE MYRISTOYLATION	
Hemolysin A (*E. coli*)	IL-1α, β precursors TNFα precursor

Myristoylation also enhances protein-protein interactions; for example, myristoylation of Gα subunits has been shown to increase their affinity for the βγ heterodimer (Linder *et al.*, 1991). The first direct evidence of the involvement of myristoylation in protein-protein interactions was noted on studies on NAP-22. An N-terminal myristoylated nonapeptide from NAP-22 bound with as high affinity as full-length protein to calmodulin (CaM) (Takasaki *et al.*, 1999). The acyl chain interacts with a hydrophobic pocket of CaM, which is usually occupied by hydrophobic amino acids of target proteins. The authors speculated that all myristoylated proteins may utilize this unique sequence to bind to CaM (Hayashi *et al.*, 2000); however, to date, only NAP-22 and HIV-1 Nef have been experimentally shown to do so (Hayashi *et al.*, 2002). Interestingly, both proteins contain the diglycyl sequence MGGXXXX, with X being basic and/or hydrophobic, and are the only proteins known to possess this starting motif.

1.3.4.1 The Two Signal Hypothesis for Membrane Binding of Myristoylated Proteins

Myristoylation facilitates membrane association of Src family tyrosine kinases such as Src and Fyn (Cross *et al.*, 1984; Buss and Sefton, 1985; Shenoy-Scaria *et al.*, 1993), G protein α subunits (Buss *et al.*, 1987; Mumby *et al.*, 1990), or retroviral Gag proteins (Henderson *et al.*, 1982; Schultz and Oroszlan, 1983; Rein *et al.*, 1986; Bryant *et al.*, 1991). Protein-bound myristate has been shown to insert hydrophobically into the lipid bilayer, and approximately 10 of the 14 carbons penetrate the hydrocarbon core of the bilayer (Sankaram, 1994; Murray *et al.*, 1998). However, the modification of protein by myristate alone does not provide sufficient binding energy to stably anchor that protein within a cellular membrane (Peitzsch and McLaughlin, 1993), and allows transient sampling of all intracellular membranes in a rapid fashion (Shahinian and Silvius, 1995).

Thus, a "second membrane-binding signal" is required for stable membrane association. A possible second signal is often a downstream polybasic domain, palmitoylation site, or protein-protein interactions (Resh, 1999). Basic amino acids form electrostatic interactions with the headgroups of the acidic phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI) that greatly enhance the membrane binding of myristoylated proteins such as Src (Buser *et al.*, 1994; Sigal *et al.*, 1994;

Murray *et al.*, 1997), MARCKS (McLaughlin and Aderem, 1995) and HIV-1 Gag and Nef (Zhou *et al.*, 1994; Welker *et al.*, 1998). Palmitoylated, myristoylated proteins include NRTKs such as Fyn, Lck and Yes and many G protein α subunits (Parenti *et al.*, 1993; Shenoy-Scaria *et al.*, 1993). Proteins utilizing protein-protein interactions include nonmyristoylated or nonpalmitoylated G protein α subunits (Hepler *et al.*, 1996; Morales *et al.*, 1998; Fishburn *et al.*, 2000).

1.3.4.2 Surface-Potential Sensor Model for Membrane Binding of Polybasic Domain-Containing Myristoylated Proteins

A polybasic second signal is intrinsic to some myristoylated proteins, and is therefore present before myristoylation occurs. Neither the hydrophobic nor the electrostatic interactions alone are sufficient to provide strong membrane binding. However, when combined, the forces synergize and provide stable membrane binding (Epand, 1997), as seen in studies involving the binding of Src N-terminal peptides/chimeras (Sigal *et al.*, 1994; McCabe and Berthiaume, 1999) and farnesylated, polybasic K-Ras4B C-terminal peptides to lipid vesicles (Leventis and Silvius, 1998). The myristoylated, polybasic sequence and full-length intact Src have been shown to discriminate strongly between lipid surfaces of differing surface charge. Two lipid bilayers differing in anionic lipid content (neutral *vs.* physiologic 33% acidic phospholipid) will show a 1000-fold preferential accumulation of the lipidated peptide/protein on the more negatively charged surface (Buser *et al.*, 1994; Sigal *et al.*, 1994). The hydrophobic effect mediated by myristate in partitioning an acylated Src peptide into membranes is 10 to 100-fold higher than the partitioning due to the basic cluster (Buser *et al.*, 1994; Murray *et al.*, 1998). Thus, it is assumed that myristate partitions into the membrane first, and acts as the first signal.

In eukaryotic cells, the inner leaflet of the plasma membrane is composed of about 30% acidic and 70% neutral phospholipids, and anionic phospholipids appear to be concentrated at the cytoplasmic face of the plasma membrane (Op Den Kamp, 1981; Gennis, 1988; Williamson and Schlegel, 1994). Thus, to target the plasma membrane, the polybasic domain in myristoylated proteins has been suggested to function as a membrane surface-potential sensor, "sensing" nonspecific electrostatic binding sites mediated by the presence of negative phospholipids such as PS, PI, PIP, and PIP_2 (Leventis and Silvius, 1998; Roy *et al.*, 2000). Although some polybasic domains have not been shown to exhibit preferential binding to particular anionic lipids (Leventis and Silvius, 1998), others, such as one found in MARCKS show specificity for PIP_2 (Rauch *et al.*, 2002). Endosomal membranes have been reported to be both depleted and enriched in PS, and may also be a potential target membrane for these proteins (Evans and Hardison, 1985; Urade *et al.*, 1988; Ciaffoni *et al.*, 2001).

1.3.4.3 Palmitate as Second Signal for Membrane Association

As will be discussed in **Section 1.4.5**, palmitoylation is believed to act in retaining a protein at a given membrane (following transient sampling mediated by myristoylation) via enzymatic modification by a palmitoyl acyltransferase (PAT) contained within that membrane. Numerous NRTKs and G protein α subunits possess this dual modification.

1.3.4.4 Protein-Protein Interactions as "Second Signal" for Membrane Association

Certain myristoylated proteins are neither palmitoylated nor contain a polybasic domain, such as the heterotrimeric G protein α subunit transducin or nonmyristoylated mutants of $G\alpha_z$ and $G\alpha_i$ (Kokame *et al.*, 1992; Neubert *et al.*, 1992; Galbiati *et al.*, 1996; Morales *et al.*, 1998; Fishburn *et al.*, 1999). Protein-protein interactions with the $\beta\gamma$ subunit direct the protein in localizing to particular membranes. Bourne and coworkers demonstrated that protein-protein interactions between α_z subunit and mitochondrially mistargeted $\beta\gamma$ controls targeting of α_z to mitochondria, implying that the protein-protein interaction may be the predominate factor (the "first signal") in specific membrane association, with lipid modifications playing a stabilizing role by increasing membrane avidity (Fishburn *et al.*, 2000).

1.3.5 Regulation of Membrane Binding of Myristoylated Proteins

1.3.5.1 Disruption of Membrane Binding Second Signal

By removing palmitate in the case of dually acylated proteins (*e.g.* NRTKs, G proteins) or phosphorylating key residues in a polybasic domain (*e.g.* Src, MARCKS), the affinity of the protein for membranes can be greatly reduced. However, protein-

protein interactions in place at the time of disruption may prevent the release of protein from membrane, but allow for increased lateral movement in the plane of the membrane, as demonstrated for Gα subunits (Huang *et al.*, 1999).

1.3.5.2 Alteration of Myristoylation Levels

The amide bond that links myristate to the N-terminal glycine is stable, and in general is present for the life of the protein (Wolven *et al.*, 1997). A few reports suggesting a demyristoylation mechanism involving the myristoylated alanine-rich C kinase substrate (MARCKS) protein exist, forming a phosphorylated demyristoylated protein when incubated with a brain cytoplasmic synaptosome fraction (Manenti *et al.*, 1994, 1995; Braun *et al.*, 2000). This deacylated product can be remyristoylated in the presence of myristoyl-CoA and NMT, demonstrating that cycles of myristoylation/demyristoylation of MARCKS can be achieved *in vitro*. Braun *et al.* also demonstrated a myristoylation-dependent N-terminal cleavage between residues Lys6 and Thr7 of MARCKS exclusively by macrophage extracts, presumably suggesting an irreversible means to remove the myristoylation signal from a modified protein (Braun *et al.*, 2000).

1.3.5.3 Myristoyl Switches

Many examples have been discovered where myristoylated proteins may exist in two conformations. In one conformation, the myristate is sequestered in a hydrophobic pocket within the protein. Upon activation/deactivation, the myristate is flipped out and becomes available for membrane binding/protein interaction. The transition between states is regulated by a mechanism known as the myristoyl switch. Proteins using this mechanism exhibit reversible membrane binding. The triggers for the switch include ligand binding, electrostatics and proteolysis (Resh, 1999).

Two examples of ligand-mediated myristoyl switch proteins are recoverin and Arf. Recoverin is a Ca^{2+}-binding protein in the retina that inhibits rhodopsin kinase and regulates the phosphorylation of photoexcited rhodopsin. In the Ca^{2+}-free state, the myristate is sequestered in a hydrophobic pocket. Binding of Ca^{2+} induces a conformation change and the myristate is exposed, allowing membrane binding (Ames *et al.*, 1997). In the case of Arf, guanine nucleotide binding regulates the exposure of the myristate residue. In the GDP-bound form, the myristoylated N-terminal α-helix is buried in a shallow hydrophobic groove. Binding of GTP triggers an expulsion of the myristoylated N-terminus and subsequent membrane binding (Goldberg, 1998).

The MARCKS protein is a PKC substrate whose membrane binding is regulated by a myristoyl electrostatic switch (McLaughlin and Aderem, 1995). Its myristate plus polybasic domain mediates binding of nonphosphorylated MARCKS to membranes. Phosphorylation of MARCKS by PKC occurs within the MARCKS basic domain, resulting in the introduction of negative charges into a positively charged region. This reduces the electrostatic interactions with acidic phospholipids and results in displacement of MARCKS from membrane to cytosol (Thelen *et al.*, 1991). The switch mechanism can be selectively impaired by replacing the N-terminal myristoylated sequence of MARCKS with a more hydrophobic, dually palmitoylated sequence from the growth cone associate protein of 43 kDa (GAP-43). It can also be disrupted by deleting the intervening sequence between the myristoyl moiety and the basic effector domain, increasing the cooperativity in membrane binding between the acyl chain and polybasic domain (Seykora *et al.*, 1996). The GAP-43/MARCKS and internal deletion chimeras remained at the plasma membrane, even when phosphorylated by PKC, and fibroblasts expressing the mutant chimeras are impaired in cell spreading, illustrating the functional importance of the myristoyl switch (Myat *et al.*, 1997).

Additional examples of myristoyl-electrostatic switches include hisactophilin, a histidine-rich actin binding protein in *Dictyostelium*. Alteration of the pH above 7 changes the charge of the histidines and triggers a conformational change and membrane binding. The N-terminal motif of Src contains PKC and PKA phosphorylation sites, and modification reduces the partitioning of model Src peptides onto lipid bilayers 10-fold (Murray *et al.*, 1998). Translocation to cytosol from plasma membrane upon stimulation/phosphorylation has been demonstrated (Walker *et al.*, 1993; Murray *et al.*, 1998). Finally, Asn2 deamidation to Asp2 of PKA catalytic subunits appears to be the exclusive pathway for introduction of an acidic residue adjacent to the myristoylated N-terminal glycine (see **Table 1.2**). This may be significant in that negative charges close to myristic acid in some proteins contribute to regulating their cellular localization (Jedrzejewski *et al.*, 1998).

Work with viral Gag proteins has uncovered a third proteolytic switch mechanism. Gag is synthesized as a polyprotein precursor (Pr55gag) that is directed to the plasma membrane by a myristate plus polybasic domain (Zhou and Resh, 1996). During viral assembly, Gag is cleaved by the viral protease, leaving an N-terminal cleavage product, p17MA, which has the same N-terminal sequence as Pr55gag, yet binds weakly to membranes. This differential binding is mediated by a p17MA C-terminal region α-helix, which in the full-length polyprotein is sequestered and unavailable to bind to the N-terminus. Upon proteolysis, the p17MA protein undergoes conformational change, and this α-helix region is believed to bind and sequester the N-terminal myristate signal, and allow for dissociation of the truncated p17 protein from the membrane. Release of p17MA from the membrane is important for viral infectivity, as this matrix protein forms a component of the preintegration complex that translocates to the nucleus upon HIV-1 infection (Gallay *et al.*, 1995).

A recent exciting discovery relates to the discovery of conclusive post-translational myristoylation of the proapoptotic molecule, BID. In some ways this may be regarded as a unidirectional proteolytic switch mechanism, as caspase 8-mediated proteolytic cleavage of p21 BID creates the myristoylated p7-p15 BID complex which activates a mitochondrial apoptotic signaling cascade (Zha *et al.*, 2000).

1.4 PALMITOYLATION

1.4.1 Historical Perspectives

Protein palmitoylation was first described in 1951 by Folch and Lees, who characterized the covalent addition of lipid to a protein, brain myelin proteolipid protein (PLP) (Folch and Lees, 1951). In the early seventies, several laboratories reported the presence of palmitic, oleic and stearic acids covalently associated via an ester linkage in highly purified preparations of PLP (Braun and Radin, 1969; Gagnon *et al.*, 1971; Stoffyn and Folch-Pi, 1971). Nearly 10 years passed before this process was rediscovered in eukaryotic cells as a post-translational modification of viral glycoproteins and subsequently shown to be a ubiquitous cellular modification (Schmidt *et al.*, 1979;

Schmidt and Schlesinger, 1979; Schlesinger *et al.*, 1980). This led to an explosion of activity in characterizing palmitoylated integral membrane proteins, such as the human transferrin receptor, rhodopsin, and human leukocyte antigens (HLA) (Omary and Trowbridge, 1981; Magee and Schlesinger, 1982; Kaufman *et al.*, 1984; O'Brien and Zatz, 1984). PAT and thioesterase enzymatic activities were initially characterized in the early 1980s in microsomal fractions using viral glycoproteins as substrates (Berger and Schmidt, 1985, 1986). Classic papers published in the 1980s demonstrated the hydroxylamine sensitivity of this cysteine thioester linkage, its posttranslational nature, the distinction between proteins modified by myristate and palmitate, and their subcellular distribution (Magee *et al.*, 1984; Magee and Courtneidge, 1985; Olson *et al.*, 1985). Endogenously synthesized radiolabeled fatty acids were also shown to incorporate into proteins (Towler and Glaser, 1986). Palmitoylation was shown not to be specific for the fatty acids incorporated. In many studies, palmitate, stearate, and oleate were shown to be substrates (Berger and Schmidt, 1984, 1985; Bizzozero *et al.*, 1986). Myristoylated proteins divided between membrane and cytosol, whereas palmitoylated proteins were almost exclusively membrane-associated at the cytoplasmic surface of the plasma membrane (Wilcox and Olson, 1987). Such localization was consistent with published reports on the subcellular localization of partially purified PAT activities, which were found to be membrane-bound (Schmidt and Burns, 1989; Berthiaume and Resh, 1995; Dunphy *et al.*, 1996).

Buss and coworkers demonstrated the first peripheral signaling molecule to be palmitoylated, Ras, near the C-terminus (Buss and Sefton, 1986). G protein α subunit and NRTK families were later found to be palmitoylated, often in conjunction with myristoylation (Linder *et al.*, 1993; Muszbek and Laposata, 1993; Paige *et al.*, 1993; Parenti *et al.*, 1993; Wedegaertner *et al.*, 1993).

The dynamic and possible regulatory nature of palmitoylation, in contrast to myristoylation and farnesylation, was suggested in pulse-chase studies which showed the half-life of [^{3}H]palmitate ranging from 20 to 180 min, far less than the half-life of the protein (Magee *et al.*, 1987; Staufenbiel, 1987, 1988). Palmitoylation of $G\alpha_s$ was found to be dynamic and regulated by activation with increased palmitate incorporation (Degtyarev *et al.*, 1993) and rapid turnover (Wedegaertner and Bourne, 1994). This incorporation and turnover of radiolabeled fatty acid was also seen in reports involving the G protein-coupled receptor (GPCR) β_2-adrenergic receptor (β_2-AR) (Mouillac *et al.*, 1992; Bouvier *et al.*, 1995b).

New types of palmitoylation motifs were discovered. An N-terminal dipalmitoylation motif was discovered in the neuronal protein, GAP-43 (Skene and Virag, 1989; Zuber *et al.*, 1989). Palmitoylation of SNAP-25 (and other cysteine string protein family members) was found to occur on an internal motif rich in cysteine residues surrounded by polar amino and carboxy amino acids (Gundersen *et al.*, 1994; Gonzalo and Linder, 1998). N-terminal cysteine amide palmitoylation was identified in the extracellular protein, human Sonic hedgehog (Pepinsky *et al.*, 1998).

A landmark paper in 1990 by Hancock *et al.* showed that membrane targeting for Ras isoforms could be mediated by the combination of two membrane association signals: farnesylation plus palmitoylation, or farnesylation plus polybasic domain (Hancock *et al.*, 1990). In the early 1990s, another role for palmitoylation/fatty acylation was identified by Resh and coworkers, who found that mitochondrial proteins could be inhibited (some at their active site) via S-acylation by fatty acyl-CoAs 8-18 carbons in length, including methylmalonyl semialdehyde dehydrogenase (MMSDH) and glutamate dehydrogenase (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). Later, carbamoyl phosphate synthetase 1 (CPS-1), another mitochondrial enzyme, was also shown to be acylated at its active site (Corvi *et al.*, 2001).

The propensity for lipid-modified proteins to be associated with biochemically derived membrane subdomains termed lipid rafts/caveolae was first shown for GPI-anchored proteins (Brown and Rose, 1992). Subsequently, the noncovalent association in lipid rafts between myristoylated and palmitoylated proteins and GPI-anchored proteins such as decay accelerating factor (DAF) in immunoprecipitation experiments in T cells was initially believed to be mediated by fatty acylation (Shenoy-Scaria *et al.*, 1993). These initial studies led to numerous investigations looking at the association of myristoylated and palmitoylated proteins with lipid rafts (see **Section 1.4.5.7**).

1.4.2 Enzymology of Palmitoylation

1.4.2.1 Palmitoyl Acyltransferase (PAT)

The identity of the enzyme(s) responsible for protein palmitoylation have not yet been firmly established, and some investigators believe there is sufficient evidence to postulate nonenzymatic palmitoylation. To date, no purified PAT activity has been successfully amenable to complete purification, sequencing and cloning analysis, although a *Drosophila melanogaster* gene product, skinny hedgehog, has been linked to palmitoylation of cholesterol-modified hedgehog proteins and presumptively identified as an acyltransferase (Chamoun *et al.*, 2001).

Partial success in PAT characterization occurred when analyzing fatty acylation of viral glycoproteins. This activity was proposed to occur in the ER (Rizzolo and Kornfeld, 1988). A PAT in the rough ER was partially purified from microsomes, revealing an integral membrane protein. No transfer occurred when microsomes were boiled or omitted. Interestingly, the Semliki Forest virus (SFV) E1 protein had to be deacylated first, as no transfer of palmitate occurred on untreated protein. The type and concentration of detergent were found to be critical for the acylation reaction, which was time- and temperature-dependent, energy requiring (ATP-specific), with palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and myristic acid (C14:0) all transferred as CoA substrates (Berger and Schmidt, 1984, 1985). Similar findings occurred when analyzing the palmitoylation of VSV G glycoprotein (Mack *et al.*, 1987). Later, a PAT activity was partially purified from the Golgi region that transfers palmitate to N-Ras. This activity was also temperature- and time-dependent, was abolished by boiling, and farnesylated N-Ras was a better substrate than unmodified protein (Gutierrez and Magee, 1991).

Enzyme activities palmitoylating NRTKs and G protein α subunits were partially purified (Berthiaume and Resh, 1995; Dunphy *et al.*, 1996). Both PATs were membrane-bound, and exhibited a preference for myristoylated substrates and for palmitoyl-CoA over other acyl-CoA substrates. The G protein PAT was enriched in plasma membrane/endosome fractions, and later shown to be enriched in cholesterol-rich low density membranes, and dispersed if incubated with methyl β-cyclodextrin (MβCD), a cholesterol-depleting agent (Dunphy *et al.*, 2001). A 70 kDa PAT was described that palmitoylates red blood cell spectrin (Das *et al.*, 1997). Using a nonprenylated *Drosophila* Ras peptide as substrate, a PAT was cloned from *Bombyx mori* (Ueno and Suzuki, 1997). This PAT consisted of two polypeptides with sequence similarity to fatty acid synthase, an enzyme catalyzing palmitate synthesis. Gelb and coworkers used Ras as a substrate and purified a PAT activity that ultimately was identified as thiolase A, an enzyme that participates in the terminal steps of β-oxidation of fatty acids (Liu *et al.*, 1996). Since thiolase is localized in organelles (mitochondria and peroxisomes) and typically catalyzes hydrolysis of palmitoyl-CoA rather than palmitate transfer to proteins, it is unlikely to be the physiologically relevant PAT. A PAT that palmitoylates dipalmitoylated GAP-43 was found to be enriched in the ERGIC and Golgi (McLaughlin and Denny, 1999). Finally, recently discovered was skinny hedgehog, a secretory pathway protein with apparent PAT activity that was shown to be essential for amino-terminal palmitoylation of sonic hedgehog (Chamoun *et al.*, 2001). Also, an ER membrane complex of Erf2p and Erf4p proteins is required for efficient palmitoylation of yeast Ras proteins and delivery to the PM (Lobo and Deschenes, 2001).

The sulfhydryl group of cysteine is a good nucleophile; under certain conditions nonenzymatic palmitoylation of proteins has been documented, both for full-length proteins ($G\alpha_o$, $G\alpha_i$, rhodopsin, PLP) and for peptides (based on the NRTK Yes) *in vitro* (Bizzozero *et al.*, 1987; O'Brien *et al.*, 1987; Duncan and Gilman, 1996; Bano *et al.*, 1998). Many of the characteristics of nonenzymatic palmitoylation are similar to those expected of a proteinaceous PAT activity, such as dependence on time, substrate concentration, and the presence of myristate on the protein/peptide substrate. However, evidence has accumulated arguing against a nonenzymatic process. First, the reaction kinetics for nonenzymatic palmitoylation appear to be too slow to account for the rapid palmitoylation and turnover seen in signaling proteins. *In vitro* transacylation of Gα subunits occurs with a time scale of tens of minutes. However, spontaneous nonenzymatic acylation of proteins *in vivo* is estimated to occur in the tens-of-hours-range, because of the existence of ACBPs that sequester long chain fatty acyl-CoAs and severely reduce their intracellular concentration and subsequently their rate of nonenzymatic transfer to protein (Leventis *et al.*, 1997). PAT incorporates palmitate into rhodopsin with higher efficiency (10 times higher initial rate) compared to autoacylation

(Veit *et al.*, 1998). Dunphy and coworkers demonstrated that PAT remains active in the presence of physiological concentrations of ACBP and acyl-CoA *in vitro* and is likely to represent the predominant mechanism of thioacylation *in vivo* (Dunphy *et al.*, 2000).

1.4.2.2 Palmitoyl Protein/Acyl Protein Thioesterases (PPT/APTs)

The initial suggestion of a deacylating enzyme was made in 1986 when Schmidt and coworkers discovered a microsomal acylprotein thioesterase activity *in vitro*, releasing free fatty acid (Berger and Schmidt, 1986). Detergent-solubilized microsomes from a variety of sources were competent in deacylating viral glycoproteins in a time-, temperature-, pH- and substrate level-dependent fashion.

Two thioesterases have been identified that deacylate Ras and Gα proteins. A 37 kDa thioesterase that depalmitoylates H-Ras *in vitro* was isolated first in 1993 (Camp and Hofmann, 1993). This enzyme, PPT1, is unlikely to mediate depalmitoylation of PM-bound proteins *in vivo* as it is localized inside lysosomes via the mannose 6-phosphate receptor-mediated pathway, modified by asparagine-linked oligosaccharides, and is secreted from COS cells (Camp and Hofmann, 1993; Camp *et al.*, 1994; Hellsten *et al.*, 1996; Verkruyse and Hofmann, 1996). Further characterization revealed that PPT1 is mutated in the lysosomal storage disease, infantile neuronal ceroid lipofuscinosis (Vesa *et al.*, 1995). Individuals with PPT1 mutations accumulate palmitoylcysteine and palmitoylcysteine-containing peptides generated from autophagy, the process where whole patches of membrane and cellular organelles, including cytoplasm, are engulfed in a vacuole, with the formation of an autophagosome. Lysosome fusion with autophagosomes delivers catabolic enzymes such as PPT1, and generates autophagolysosomes (Lu *et al.*, 1996; Dawson *et al.*, 1997; Hofmann *et al.*, 1997; Cho *et al.*, 2000). In neurons, PPT1 is localized to synaptosomes and synaptic vesicles, not to lysosomes, and may play a role in synaptic functioning (Lehtovirta *et al.*, 2001). PPT1 overexpression can significantly reduce [^{3}H]palmitate incorporation into cellular lipids, in particular palmitoyl-CoA, but has no effect on ecNOS palmitoylation (Michel and Michel, 1997). A homologous protein, incorrectly named PPT2, will only depalmitoylate palmitoyl-CoA, not palmitoylated proteins (Soyombo and Hofmann, 1997; Aguado and Campbell, 1999).

A second acyl protein thioesterase (APT1) that depalmitoylates Gα subunits and Ras has been cloned (Duncan and Gilman, 1998). APT1 had originally been cloned as a lysophospholipase (Sugimoto *et al.*, 1996), although the enzyme clearly prefers palmitoylated protein substrates to lipid substrates. Immunocytochemical studies localized the protein to the cytosol, ER and nuclear envelope; the significance of this as it pertains to deacylating palmitoylated proteins is unknown at this time (Wang *et al.*, 2000). Recently, APT1 was found to depalmitoylate certain palmitoylated proteins (ecNOS and $G\alpha_s$), but not others (caveolin), *in vivo* and *in vitro*. Overexpression of APT1 accelerated the depalmitoylation of its substrates (Yeh *et al.*, 1999). Furthermore, certain viral glycoproteins were rapidly depalmitoylated by APT1 (VSV G, influenza HA, SFV E2), whereas others were resistant to the activity (SFV E1) (Veit and Schmidt, 2001).

1.4.2.3 Substrate Specificity

The preferred substrate for palmitoylation appears to be palmitoyl-CoA (Berthiaume and Resh, 1995; Dunphy *et al.*, 1996). However, several proteins have also been shown to incorporate other long chain fatty acids, including palmitoleate (C16:1*n*-9) (Casey *et al.*, 1994), stearate (C18:0) (Veit *et al.*, 1996; Van Cott *et al.*, 1997), oleate (C18:1*n*-9) (Bizzozero *et al.*, 1986; Muszbek *et al.*, 1999) and *cis*-5,8,11,14,17-eicosapentaenoate and arachidonate (C20:4 (5,8,11,14)) (Hallak *et al.*, 1994; Laposata and Muszbek, 1996). Stearate and oleate are by far the most common alternate substrates (Bizzozero *et al.*, 1986). It was also shown that supplementing the growth media of cultured cells with alternate unsaturated fatty acids led to incorporation into acylated proteins (Muszbek *et al.*, 1999; Liang *et al.*, 2001). Thus, '*S*-acylation' provides a more accurate description of this type of modification (Casey, 1995). Whether acylation with different fatty acids reflects regional acyl-CoA pool distribution or affects protein function is not known. Pool and Thompson suggested that the decreased specificity of palmitoylation may also stem from the fact that this modification is frequently acting as a second signal in localizing lipidated proteins to membranes. The addition of this second lipid group to trap a protein in a membrane would not be as dependent on the absolute hydrophobicity of that single modification, and it would be more efficient to use whichever group is more abundant (Pool and Thompson, 1998). Significant levels of acyl-CoA hydrolases exist within the cytosol, mitochondria, and on ER membranes (Lehner and Kuksis, 1993; Svensson *et al.*, 1995; Broustas *et al.*, 1996; Soyombo and Hofmann, 1997). Conversely, acyl-CoA synthetase (ACS) activities have been found associated with a variety of membranes, including PM, endosomes, and ER (Sleeman *et al.*, 1998; Gargiulo *et al.*, 1999; Black *et al.*, 2000). Thus, localized production and sequestration in the membrane may be the mechanism by which acyl-CoA substrate is generated for PAT, or other acyl-CoA_utilizing enzymes to transfer. It has been speculated that the efficient partitioning of different long chain acyl-CoAs with varying degrees of unsaturation into membranes may allow for a number of different substrates to be incorporated into *S*-acylated proteins. Acyl-CoAs are amphipathic detergent-like molecules and partitioning into membranes markedly affects membrane integrity (Faergeman and Knudsen, 1997). This suggests the importance of regulating the concentration of these species intracellularly, possibly through ACBP binding or other mechanisms.

1.4.2.4 Stoichiometry of Palmitoylation

In contrast to myristoylation, where acylation efficiency is essentially 100% (Farazi *et al.*, 2001), the stoichiometry of palmitoylation has been more difficult to ascertain. Nonenzymatic palmitoylation of cysteinyl-containing lipotetrapeptides containing a myristoylated glycine to high stoichiometry *in vitro* was reported (Quesnel and Silvius, 1994). Using NIH-3T3 cells labeled to steady state with the iodinated palmitate analogue IC16, Resh and coworkers estimated that overexpressed wild-type (WT) Fyn was 50% palmitoylated (Wolven *et al.*, 1997). Jones and coworkers found that approximately 77% of the endogenous Gα_s in COS cells and 70% in S49 lymphoma cells were palmitoylated. The fraction of Gα_s that was modified did not change after treatment with isoproterenol, a β_2-AR agonist that causes turnover of palmitate on Gα_s. These results suggest that receptor activation of Gα_s caused a rapid turnover of palmitate to maintain most of Gα_s in its palmitoylated form (Jones *et al.*, 1997). The membrane-spanning domain of neurofascin, a cell adhesion molecule, is constitutively palmitoylated at Cys1213 at close to 1:1 molar stoichiometry, and does not turn over for the life of the protein (Ren and Bennett, 1998). Also, palmitate incorporation into caveolin was unexpectedly blocked by the protein synthesis inhibitors cycloheximide and puromycin, and pulse-chase experiments showed that caveolin-1 palmitoylation was essentially irreversible since release of [^{3}H]palmitate was not significant even after 24 h (Parat and Fox, 2001). Thus, both stable and dynamically palmitoylated proteins appear to be predominately palmitoylated *in vivo*.

1.4.2.5 Protein Consensus Sequences

The elucidation of consensus signal(s) for palmitoylation has been more difficult. Previous reviews on the subject categorized palmitoylated proteins into four categories (**Table 1.4**) (Resh, 1996, 1999).

Type I sequences consist of PM receptors and other membrane proteins that are typically palmitoylated on one or more cysteine residues located adjacent to or just within the transmembrane domain (TMD). The only common characteristic of the palmitoylated cysteine residues is their location on the cytoplasmic side near TMDs, or in the TMD itself (Schlesinger *et al.*, 1993). Schweizer *et al.*, who studied the structural requirements for the ER protein p63, concluded that palmitoylation occurs without a primary sequence motif: only the six amino acid spacing between the cysteine to be palmitoylated and the TMD allowed efficient palmitoylation. The identities of residues surrounding the cysteine and the TMD were not critical for palmitoylation (Schweizer *et al.*, 1995a). A recent study by ten Brinke *et al.* studying lung surfactant protein C suggests that its palmitoylation does not rely on specific sequence motifs, but more on the probability that the cysteine is in the vicinity of the membrane surface (ten Brinke *et al.*, 2002).

In contrast to these reports, other investigators have identified sequence motifs felt to be critical for acylation occurrence and identity. Although suggestive of an enzymatic recognition sequence, it remains plausible that these "motifs" are merely indicative of protein conformations competent for thiol acylation via a pool of membrane-bound acyl-CoA. When cysteines were inserted by mutagenesis into a non-acylated fusion glycoprotein of Sendai virus at a spacing of 6 to 10 amino acids from the TMD, this was not sufficient to cause palmitoylation of this cysteine (Ponimaskin and Schmidt, 1995). Thus, the presence of a cysteine residue in the region of the TMD in

TABLE 1.4: Palmitoylated Proteins

TYPE I: INTEGRAL MEMBRANE OR CYTOSKELETAL PROTEINS	
Cytoskeletal Proteins	**Hematopoietic Cell Proteins**
Actin (slime mold) Ankyrin Band 4.1 Band 4.2 Lens α-crystallin Microtubule binding protein (*T. brucei*) Tubulin Vinculin	β-2 microglobulin Band 3 anion exchanger (AE1) CD4, 8β, 9, 44 Glycophorin gp40 T-cell protein HLA antigen B7, DR heavy chains, D invariant chain LAT Iaα, β Kx, p55 Platelet glycoproteins IX, Ibβ, IV P-selectin Sialo-gp2, gp3 and gp3Pr proteins vWF receptor
Cell Surface/Endomembrane Receptors/Channels	**Viral Proteins**
Adenylyl cyclase Ca^{2+} channel β_{2a} subunit Caveolin-1 CD36/SR-B1 CD39 ectonucleotidase CD-MPR Endothelin converting enzyme (ECE) Galactosyltransferase GI epithelial A33 antigen GLUT1 glucose transporter IgE receptor IL-2 receptor Insulin, IGF-1 receptors Lens fibre protein MP26 (MIP26) Ligatin Lymphoma proprotein convertase MAG Mannosidase II Myelin P0 glycoprotein Na^{+} channel α subunit Neural cell adhesion molecule (NCAM) Neurofascin p62/63 (ER/ERGIC protein) Phospholipid scramblase Proteolipid protein (PLP)/DM-20 proTNF Retinoid-binding protein Sm25 (*S. mansoni*) Stomatin Synaptobrevin 2 (yeast Snc1, Snc2) Synaptotagmin 1 TGFα precursor Transferrin receptor	Adenovirus types 2, 5, 12 E1b, ADP Avian leukosis virus $gp35^{env}$ Canine distemper F Coronavirus L9 E2 Epstein Barr LMP1, LMP2 Fowl Plague HA Friend murine leukemia env HIV-1 gp160 (env) HSV gE Influenza A HA, HA_2, H1 Influenza B HA Influenza C HEF I, HEF II, CM2 LaCrosse G1, G2 Measles F Moloney murine leukemia R peptide Mouse hepatitis E2 Mumps paramyxovirus HN, F Newcastle F0, F1 Rhabdovirus G protein Rift valley fever virus G2 Ross River E1 Rous sarcoma virus $gp35^{env}$ Rubella E1, E2 Semliki Forest nsP1, E1, E2, p62 Simian virus 5 HN (hemagglutinin-neuramidase) Sindbis virus E1, E2 6K, nsP1 SIV env Spleen focus-forming $gp65^{env}$ SV40 large T antigen Togavirus E, E2 Vaccinia p37, 14, 17, 26, 41, and 92 VSV G

TYPE I, CONTINUED…	TYPE II: PRENYLATED, PALMITOYLATED PROTEINS
G-protein coupled receptors	
α_{2A}, β_2 adrenergic A1, A3 adenosine Acetylcholine Bradykinin B1, B2 CCR5 chemokine D2S Dopamine D_1, D_{2L} Endothelin A, B FSH Gastrin releasing peptide GluR6 (kainate) Histamine H2 LH/hCG mGlu R4 Mu opioid Neurokinin B Rhodopsin Serotonin Serotonin 5-HT_{1B}, 5-HT_{4A} Somatostatin 5 Thyrotropin Vasopressin V1a, V2	H-Ras N-Ras K-Ras4A Paralemmin 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase) Yeast RAS1, RAS2 Yeast G protein γ subunit Ste18p

TYPE III: N-, C-TERMINAL AND INTERNALLY PALMITOYLATED PERIPHERAL PROTEINS	TYPE IV: MYRISTOYLATED, PALMITOYLATED PROTEINS
G Protein α Subunits	**Src Family Tyrosine Kinases**
$G\alpha_s$ $G\alpha_q$ $G\alpha_{11, 12, 13, 16}$ $XL_{\alpha s}$	Fgr Fyn Hck Lck Lyn Yes Yrk
Other Signal Transduction Proteins	**G Protein α Subunits**
CHIC2 Cysteine string proteins GAD-65 GAIP GAP-43 GRIP1 GRK4, 6 G_z GAP PI4K family PKC PLD1 PSD-95 and PSD-93 RB3 RET RGS1 RGS4, 5, 7, 10 , 16 Rlk/Txk SCG-10 SNAP-25A and B Sprouty-1,2 Syndet	$G\alpha_{i1,2,3}$ $G\alpha_o$ $G\alpha_z$ Yeast Gpa1, 2 **Miscellaneous** ecNOS RP2 retinal protein HASPB (*L. major*) AKAP15, 18 FCaBP (*T. cruzi*) RasGRP2 Ara6 (*A. thaliana)* PPEF (*C. elegans*) GRASP55 OSCPK2 (rice kinase) Vac8p (*S. cerevisiae)* Psr1p,2p (*S. cerevisiae)*

SECRETED PALMITOYLATED PROTEINS	CYSTEINE AMIDE PALMITOYLATION
Apolipoprotein B Interphotoreceptor retinoid binding protein (IRBP) Sonic hedgehog Lung surfactant protein (SP-C) Caveolin-1 Salivary mucus glycoprotein Gastric mucus glycoprotein Adipocyte differentiation related peptide (ADRP) Xanthine oxidase Butyrophilin	Sonic hedgehog family **MITOCHONDRIAL PALMITOYLATION** MMSDH CPS-1 Glutamate dehydrogenase

BACTERIAL PALMITOYLATION	MISCELLANEOUS
Adenylate cyclase toxin (*B. pertussis*) Hemolysin (*E. coli*) Nitrogenase (Cyanobacteria)	Yeast protoporphyrinogen oxidase

certain proteins is not sufficient for palmitoylation to occur. To support this, Belanger *et al.* have demonstrated an essential role of adjacent basic residues, a promoting effect of hydrophobic residues, and inhibition by acidic residues in nonenzymatic palmitoylation studies of β_2-AR peptides *in vitro* (Belanger *et al.*, 2001). By computer-assisted alignment of known palmitoylated TMD proteins, Grosenbach and coworkers formulated a conserved acceptor motif of integral membrane proteins:

$$\mathrm{TMDX_{1\text{-}12}AAC(C)A}$$

in which TMD stands for transmembrane domain, X for any amino acid, A for aliphatic amino acid, and (C) for potential second cysteine (Grosenbach *et al.*, 1997). A GPCR sequence analysis (59 sequences) by Schulein *et al.* revealed a dihydrophobic pair (containing Leu, Ile, Phe, Val, or Met) 10-15 residues from the end of the seventh transmembrane domain, with known and putative palmitoylated cysteine residues 1-6 residues C-terminal to this dihydrophobic pair in 80% of the sequences (Schulein *et al.*, 1998). Bouvier proposed the following consensus motif from this work:

$$\mathrm{F/Y(X)_{0\text{-}4}B(X)_{0\text{-}4}C}$$

where F is Phe, Y is Tyr, B is a hydrophobic residue, X for any amino acid, and C is the palmitoylated cysteine (Bouvier *et al.*, 1995a). Ponimaskin and Schmidt suggested a common motif of one or two palmitoylated cysteine residues located either 1) up to 10 amino acids C-terminal to the TMD, or 2) within the TMD within 6 amino acids of the cytoplasmic border (Ponimaskin and Schmidt, 1995). These investigators also found that the length and composition of the TMD as well as the cytoplasmic tail appear to influence the choice of fatty acid for acylation. Integral membrane proteins with short basic cytoplasmic tails are primarily stearoylated, while longer tail proteins are palmitoylated (Ponimaskin and Schmidt, 1995; Reverey *et al.*, 1996; Veit *et al.*, 1996; Ponimaskin and Schmidt, 1998).

Type II proteins include members of the Ras family that are modified with a farnesyl moiety in their C-terminal CAAX-box, with subsequent palmitoylation of adjacent cysteine residues (Hancock *et al.*, 1989).

A third group of peripheral proteins (Type III) is palmitoylated at cysteine residues near, but not at, the N- and C-termini. A critical addition to this category is the inclusion of internally palmitoylated peripheral proteins, such as SNAP-25 and the cysteine string protein family. A hydrophobic consensus sequence for postsynaptic density protein of 95 kDa (PSD-95) and GAP-43 (and possibly Gα_q) was identified to consist of five consecutive hydrophobic amino acids (containing the two palmitoylated cysteine residues), with 2-3 adjacent downstream basic residues (Liu *et al.*, 1993; Wedegaertner *et al.*, 1993). Conservative mutation of any of these five residues, including the cysteines, preserves palmitoylation, whereas mutations to a hydrophilic amino acid prevent palmitoylation. Interestingly, insertion of 6 amino acids (VSKSGS) after the initiator Met does not influence the efficacy of palmitoylation of PSD-95 (El-Husseini *et al.*, 2000). Some Type III proteins, like SNAP-25, appear to have a complex membrane association domain involving α-helical structure, which allows for recruitment and binding to a membrane-bound physiological partner (syntaxin-1) and secondary palmitoylation once at the vesicle/plasma membrane (Veit, 2000).

Type IV proteins are dually fatty acylated with myristate and palmitate and a majority contain the consensus sequence Met-Gly-Cys at their N-termini. A number of miscellaneous dually acylated proteins, from a variety of organisms and subcellular locales, contain palmitoylated cysteines slightly farther downstream, as in the case of AKAP15/18, HASPB, RasGRP2, and Vac8p, or much farther downstream, as in Psr1p/Psr2p and ecNOS (**Table 1.4**).

1.4.3 Dynamic Regulation of Palmitoylation

A number of proteins have the acyl moiety turning over more rapidly than the polypeptide backbone (Omary and Trowbridge, 1981; Staufenbiel, 1987). The half-life of palmitate covalently attached to proteins varies from as little as 20 minutes for N-Ras (Magee *et al.*, 1987) to > 24 h for caveolin-1 and neurofascin (Ren and Bennett, 1998; Parat and Fox, 2001). The fact that palmitate can be metabolically released from palmitoylated proteins may be of biological importance, because it allows for regulation of this hydrophobic modification. Both biological function and subcellular location could be potentially controlled by acylation and deacylation (Schmidt, 1989; McIlhinney, 1990). However, progress in this area has been slow because of the relative instability of the modification, isotopic dilution by the large pool of cellular palmitate and nonequilibrium conditions, and a lack of acylation and deacylation enzymes that have been isolated or cloned as pure molecular species (Mumby, 1997).

It is well established that activation of $G\alpha_s$ through stimulation of the β_2-AR results in increased palmitate turnover (Degtyarev *et al.*, 1993; Mumby *et al.*, 1994). Kinetic studies have indicated that the most likely explanation is that the agonist promotes depalmitoylation of $G\alpha_s$ (Wedegaertner and Bourne, 1994). $G\alpha_s$ activation leads to dissociation of $G\alpha_s$ from $\beta\gamma$ and depalmitoylation of $G\alpha_s$ by a thioesterase (Wedegaertner and Bourne, 1994; Iiri *et al.*, 1996). A mechanistic hypothesis for the increase in turnover is that the activated protein is a better substrate for a constitutive palmitoylthioesterase (Mumby, 1997). This was supported in studies by Yeh *et al.*, who showed that depalmitoylation of ecNOS by APT1 was found to be potentiated by CaM, an allosteric activator of ecNOS (Yeh *et al.*, 1999). Following GTP hydrolysis, $G\alpha_s$ reassociates with $\beta\gamma$ and is rapidly repalmitoylated. The stoichiometry of $G\alpha_s$ palmitoylation does not change upon activation; GPCR stoichiometry has not been assessed (Jones *et al.*, 1997). Initial reports suggested depalmitoylation may lead to G protein-GPCR dissociation with release of $G\alpha_s$ into the cytosol (Wedegaertner *et al.*, 1996); later reports do not support this and suggest that $G\alpha$ subunits remain membrane associated during activation and depalmitoylation (Huang *et al.*, 1999). Supporting this notion, agonist binding induced the rapid translocation and depalmitoylation of $G\alpha_q$ from plasma membrane to intracellular membranes via endocytosis; this spatial separation may act as a means of receptor desensitization (Arthur *et al.*, 1999).

Moffet *et al.* showed that the palmitoylated Cys341 of the β_2-AR modulates phosphorylation of the receptor by PKA and β-adrenergic receptor kinase (βARK) (Moffett *et al.*, 1996; Moffett *et al.*, 2001). Thus, depalmitoylated receptor is susceptible to desensitization and down regulation through receptor phosphorylation and G protein uncoupling, via receptor endocytosis and degradation. Recent work by Bouvier and colleagues demonstrated for the first time that depalmitoylation and repalmitoylation occur during distinct phases of the GPCR-G protein activation/inactivation cycle (Loisel *et al.*, 1996; Loisel *et al.*, 1999). They also showed that agonist stimulation promotes the rapid depalmitoylation of both β_2-AR GPCR and Gα_s. The extent of depalmitoylation is directly proportional to the intrinsic activity of the agonist used. Thus, dynamic palmitoylation can act as a regulatory switch that affects the potency of a signaling cascade, via effects on the GPCR/G protein interaction, the intrinsic activities of these proteins, and their location or competency for endocytosis/degradation. Reversible palmitoylation also occurs with NRTKs, and may play a role in regulating the presence of these proteins in lipid rafts (Paige *et al.*, 1993; Wolven *et al.*, 1997).

Dynamic active site fatty acylation may regulate mitochondrial metabolism, as suggested for MMSDH, glutamate dehydrogenase, and CPS-1. All are mitochondrial enzymes involved in substrate utilization for energy production, and nitrogen balance. Inhibition of these proteins by long-chain fatty acyl-CoAs could reduce amino acid degradation and urea secretion, thereby contributing to nitrogen sparing during starvation (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994; Corvi *et al.*, 2001). Fatty acylation may be nonenzymatic in mitochondria, owing to the high levels of fatty acyl-CoA substrate that can be generated during normal metabolic function, and the absence of any ACBP in this organelle (Faergeman and Knudsen, 1997; Corvi *et al.*, 2001). Mitochondrial thioesterases are known to exist (Lee and Schulz, 1979; Gross, 1984; Svensson *et al.*, 1995; Poupon *et al.*, 1999), and thus acylation/deacylation of these proteins may be a key regulatory feature to control substrate flux through amino acid degradation versus fatty acid β-oxidation.

1.4.4 Subcellular Localization of Palmitoylated Proteins

While the vast majority of palmitoylated proteins are primarily associated with the plasma membrane, they have also been localized to membranes of endosomes/synaptic vesicles (Gundersen *et al.*, 1994; Lane and Liu, 1997), clathrin-coated vesicles (De Vries *et al.*, 1998), the rough ER (Schweizer *et al.*, 1995b), Golgi apparatus (Liu *et al.*, 1997), mitochondrial matrix and outer mitochondrial membrane (Rebollo *et al.*, 1999; Corvi *et al.*, 2001), cytoskeleton (Zambito and Wolff, 1997), and the extracellular milieu (Pepinsky *et al.*, 1998; Denny *et al.*, 2000). Palmitoylation, *per se*, does not appear to act as a specific membrane targeting feature based on these findings, but rather a means to retain a protein at these membrane sites (Pool and Thompson, 1998).

1.4.5 Functions of Palmitoylation

1.4.5.1 Involvement in Membrane Binding/Retention

The function most frequently associated with palmitoylation of soluble polypeptides is to facilitate membrane association, presumably by direct insertion of the fatty acid into the lipid bilayer. Palmitoylation appears to be confined to proteins associated with cellular membranes. A number of cytoskeletal proteins (**Table 1.4**) are palmitoylated, but it is generally believed that the palmitoylated population of these molecules is found at the interface of the cytoskeletal elements with cellular membranes (Dunphy and Linder, 1998).

An exposed fatty acid in covalent linkage promotes interaction of the acylprotein with a hydrophobic environment in its vicinity (Schmidt, 1989). Chemical acylation of IgG and α-bungarotoxin stably anchors them to liposomes (Huang *et al.*, 1980; Babbitt and Huang, 1985). Monoacylated ribonuclease A, normally a nonlipidated protein, bound to phospholipid, and the association level increased with the acyl chain length to reach a maximum for C16:0 (Roy *et al.*, 2001).

1.4.5.1.1 Kinetic Bilayer Trapping Model

As mentioned previously (Section 1.3.4.1), myristoylated proteins transiently associate with and move between intracellular membranes. Myristoylated or farnesylated peptides randomly and rapidly diffuse between unilamellar lipid vesicles ($t_{1/2} < 1$ min) (Silvius and l'Heureux, 1994). Thus, singly myristoylated proteins are predicted to be exquisitely sensitive to other properties of the protein that may encourage membrane association. Proteins that reach a membrane with an appropriate trapping receptor (*e.g.* plasma membrane PAT) leads to the addition of a second lipid (palmitate), and the protein becomes a long-lived resident of the membrane where the second lipid modification occurred because of increased hydrophobicity and the slower kinetic off rate from membranes of the dual fatty acid anchor. In combination with a myristoyl or farnesyl group, palmitoylated peptides are effectively anchored to a bilayer ($t_{1/2} > 70$ h)

(Shahinian and Silvius, 1995). Thus, the membrane localization of these proteins is maintained by a kinetic trapping mechanism based on palmitoylation of the protein at the membrane.

Several lines of evidence support this bilayer-trapping model. A plasma membrane PAT has been identified which can trap the substrate in the same membrane (Dunphy *et al.*, 1996). *N*-myristoylated peptides equilibrate into cells, associate with all cellular membranes, and are rapidly extractable with albumin. In contrast, Myr-Gly-Cys peptides are specifically palmitoylated at and remain bound to the plasma membrane, and are resistant to albumin extraction (Schroeder *et al.*, 1996). This was also shown for palmitoylated N-Ras peptides, which have a different permanent lipid anchor (farnesyl *vs.* myristate) yet display the same characteristics of membrane association as the myristoylated peptides (Schroeder *et al.*, 1997). The Myr-Gly-Cys motif has been shown to promote rapid palmitoylation and plasma membrane anchoring (within 5 min) of Fyn following synthesis and myristoylation on soluble ribosomes (van't Hof and Resh, 1997).

1.4.5.1.2 The Two Signal Hypothesis of Membrane Binding

The kinetic bilayer-trapping model best describes the membrane association of dually lipidated proteins/peptides. In theory, a single palmitoyl group should be sufficient for membrane association of small acylated amino acids or peptides. This was demonstrated experimentally with acylated peptides of varying fatty acid chain length, with myristate providing barely enough binding energy to associate with membranes, and palmitate providing a much stronger association (Peitzsch and McLaughlin, 1993; Shahinian and Silvius, 1995). However, a study by Pool and Thompson speculate that this may not be the case for full-length proteins (Pool and Thompson, 1998). Biophysical experiments using full-length acylated proteins demonstrated that none of the single lipid modifications observed to date provides enough binding energy to stably anchor any protein to a biological membrane, although palmitoyl and geranylgeranyl groups provide significantly more binding energy than myristoyl or farnesyl groups. In support of these *in vitro* observations, there are very few reports of singly palmitoylated, nonmyristoylated peripheral proteins in the literature.

Membrane-associating signals can be coupled to promote specific membrane association. This two-signal hypothesis was first described for Ras proteins. Multiple lipidation occurred at the C-terminus of Ras isoforms H-Ras and N-Ras, but not K-Ras. K-Ras was found to have an extensive polybasic domain adjacent to the farnesylated C-terminal cysteine. A "second signal" consisting of a polybasic domain or palmitoylation site was required for stable plasma membrane association of these Ras isoforms (Hancock *et al.*, 1990). Many of these signals have been found to be interchangeable; for example, nonprenylated Ras that is N-terminally myristoylated maintains membrane binding and palmitoylation (Cadwallader *et al.*, 1994). A polybasic domain restores function of *Saccharomyces cerevisiae* Ras2p and H-Ras if the prenylation sites are removed but palmitoylation sites maintained (Mitchell *et al.*, 1994; Booden *et al.*, 1999). However, a novel mutant combining N-terminal myristoylation and C-terminal farnesylation in the absence of palmitoylation was mistargeted to intracellular membranes, illustrating that two "weak" membrane-associating first signals are not sufficient for stable membrane association and proper localization as assessed by subcellular fractionation and immunolocalization (Cadwallader *et al.*, 1994).

In cases where the second signal is palmitoylation, the first signal may simply serve to allow transient interaction with membranes. This may be a signal other than myristate. For example, inhibition of myristoylation with 2-hydroxymyristate specifically inhibited the acylation of Lck and $G\alpha_{i1}$ with $[^3H]$myristate without affecting its labeling with $[^3H]$palmitate and membrane binding, implying that other membrane-targeting functions must have compensated for the loss of myristoylation. In this case, overexpression of $\beta\gamma$ subunits was found to rescue the palmitoylation and membrane binding of these proteins (Paige *et al.*, 1993; Galbiati *et al.*, 1996). Overexpression of $\beta\gamma$ to levels comparable to overexpressed α subunit can substitute for myristoylation of $G\alpha_{i1}$ and allow palmitoylation (Degtyarev *et al.*, 1994). This protein-protein interaction hypothesis has been supported by Bourne and colleagues, who showed that in $G\alpha_z$, myristoylation is normally the first signal for wild-type $G\alpha_z$. In a myristoylation-defective $G\alpha_z$ mutant, $\beta\gamma$ can substitute for myristoylation, and the prenyl group of γ and protein-protein interactions between α and $\beta\gamma$ act as the first signals for membrane association of $G\alpha_z$. Similarly, a myristoylated, palmitoylation-defective mutant $G\alpha_z$ localizes to the PM in the presence of overexpressed βγ. An elegant mistargeting experiment revealed that a mutant βγ containing an outer mitochondrial localization signal sequesters wild-type palmitoylated Gα_z at the mitochondrial surface, and not at the PM (Fishburn *et al.*, 2000). However, the authors did not assess the palmitoylation status of the mistargeted Gα subunits in that study, leaving open the possibility that the mitochondrially associated Gα_z was not palmitoylated (Bourne, 2002). In spite of this, the study suggested that protein-protein interactions may play a significant role in targeting peripheral lipidated proteins to a given membrane, while lipid modifications may stabilize interactions of proteins with membranes and with other proteins. A summary of the different signal combination used by lipidated proteins in membrane association can be seen in **Table 1.5**.

1.4.5.2 Involvement in Membrane Fusion/Vesicular Trafficking

A role of palmitoylation in viral membrane fusion was proposed by Schmidt and coworkers (Schmidt and Schlesinger, 1979, 1980; Schmidt, 1982; Schmidt and Lambrecht, 1985; Lambrecht and Schmidt, 1986). The fusion of viral and cellular membranes by viral spike glycoproteins was drastically impaired after release of palmitate by hydroxylamine. However, there are a few enveloped viruses that express surface proteins that lack conserved cysteine residues near/in TMDs, do not incorporate radiolabeled fatty acid and still enter host cells by membrane fusion, implying that the role of palmitoylation in membrane fusion may be complex.

Glick and Rothman first suggested a role for palmitoylation in membrane trafficking by demonstrating that vesicular transport between the *cis* and *medial* stacks of the Golgi is stimulated by palmitoyl-CoA (Glick and Rothman, 1987). Further studies showed that palmitoyl-CoA is required both for budding and fusion of transport vesicles, and an acylation reaction is the likely mechanism, as a nonhydrolyzable palmitoyl-CoA analogue inhibited both processes (Pfanner *et al.*, 1989; Pfanner *et al.*, 1990; Ostermann *et al.*, 1993). This may be related to work on endophilin I, a presynaptic protein that binds to dynamin, a GTPase that is implicated in endocytosis and recycling of synaptic-like

Table 1.5: "Two Signal" Hypothesis for Membrane Localization of Lipidated Proteins

Protein Examples	First Signal	Second Signal*	Location
NRTKs Fyn, Yes, Lck *G Proteins* $G\alpha_i$, $G\alpha_o$, $G\alpha_z$	Myristate	Palmitate	PM + endosomes
$G\alpha_{s, q, 11, 12, 13}$ (Mutant G2A $G\alpha_{i,o,z}$)	Protein-protein interaction (βγ)	Palmitate	PM + endosomes
GAP-43, $G\alpha_s$	Protein-protein interaction ± Polybasic Domain	Palmitate	PM ± Golgi/TGN
$G\alpha_t$	Myristate	Protein-protein interaction (βγ)	PM
Src, MARCKS	Myristate	Polybasic Domain	PM, endosomes, microtubules, actin
K-Ras	Farnesyl	Polybasic Domain	PM, endosomes microtubules, ER
H-Ras, N-Ras	Farnesyl	Palmitate	PM, Golgi, ER
SNAP-25	Protein-protein interaction (Syntaxin)	Palmitate	PM, synaptic vesicles

* Protein-protein interactions may contribute as a membrane-associating signal to all protein examples in varying degrees. This was omitted from the chart for clarity.

microvesicles. Endophilin I exhibits lysophosphatidic acid acyl transferase activity, and endophilin-I-mediated microvesicle formation requires the transfer of the unsaturated fatty acid arachidonate to lysophosphatidic acid, converting it to phosphatidic acid. Palmitoyl-CoA is also a substrate, but inhibits the budding of these particular vesicles. This may be related to the fact that arachidonyl-CoA, a 'cone-shaped' lipid, would be enriched in the negative-curvature neck region of a bud, whereas palmitoyl-CoA is an inverted cone-shaped lipid inducing positive curvature in membranes. Endophilin I is believed to induce negative membrane curvature by converting an inverted-cone-shaped lipid to a cone-shaped lipid in the cytoplasmic leaflet of the bilayer at the neck of the budding vesicle. Palmitoylation of peripheral proteins may convert them into 'inverted cone-shaped' proteolipid structures that promote positive curvature of membranes and their association with the cytoplasmic surface of budding vesicles (Schmidt *et al.*, 1999). This conclusion is supported experimentally by Escriba and coworkers, who demonstrated that the binding of $G\alpha_i$ to liposomes was promoted by the addition of nonlamellar promoting (*i.e.* cone and inverted cone lipids and cholesterol) lipid species (Escriba *et al.*, 1997). To identify an acylated protein that might act as a palmitate donor/acceptor during vesicular transport, protein acylation was examined in cells undergoing mitosis, when vesicular transport ceases (Warren, 1993). A 62-kDa protein was hyperacylated only in mitotic cells (Mundy and Warren, 1992). It was suggested by Mundy that this palmitoylated p62 protein may be a critical fission protein for budding of coated vesicles/anterograde transport from the ER (Mundy, 1995). To conclude, whether a proteinaceous or lipid acceptor of palmitate/related fatty acid (or both) is mediating vesicular trafficking is still an area of debate.

Progress in this area of palmitoylation function was quiescent until the discovery of Vac8p, a yeast protein required for vacuole inheritance. This protein is targeted to the cytosolic side of the vacuolar membrane via myristoylation and palmitoylation, and plays a role in vacuolar morphology and protein targeting to the vacuole (Fleckenstein *et al.*, 1998; Wang *et al.*, 1998). Palmitoylation is essential for vacuole inheritance, and Vac8p becomes palmitoylated when isolated yeast vacuoles are incubated under conditions that allow homotypic membrane fusion. Two recent reports clearly identify palmitoyl CoA-dependent membrane fusion reaction as being related to palmitoylation of a target protein. Vac8p is initially associated with the *cis*-SNARE complex, is palmitoylated after Sec18p (NSF) priming, and the palmitate label remains stable throughout the fusion reaction. A lack of palmitoylation allows for release from this SNARE-bound vacuole membrane (Veit *et al.*, 2001; Wang *et al.*, 2001). It appears that dual acylation of Vac8p is needed for vacuole fusion, and may enrich the protein in a defined membrane patch that promotes bilayer mixing, as seen in intervacuolar membrane patches. A similar situation may exist in plants. Ara6, a unique myristoylated and palmitoylated Rab GTPase found in *A. thaliana*, is distributed primarily in endosomes due to N-terminal acylation, nucleotide binding, and a C-terminal hypervariable region, and is believed to play a role in endosomal fusion in these cells (Ueda *et al.*, 2001).

1.4.5.3 Involvement in Integral Membrane Protein Function

In contrast to hydrophilic peripheral proteins, palmitoylated integral membrane proteins do not require covalently bound palmitate for membrane attachment. Ponimaskin and Schmidt asked the simple question, "Is the presence of one or several cysteine residues located within the cytoplasmic border region of a transmembrane protein *alone* sufficient to cause acylation?" Work on several viral glycoproteins has shown that this is not the case, lending support to the enzymatic requirement for protein palmitoylation, and a functional role for this modification (Ponimaskin and Schmidt, 1995, 1998). However, it is possible that these protein substrates adopt a particular helical or similar conformational structure that runs parallel to the membrane surface.

1.4.5.3.1 Dynamic Palmitoylation of Integral Membrane Proteins

As mentioned previously, a 62 kDa ER protein was hyperacylated only in mitotic cells, implying a dynamic nature to the modification (Mundy and Warren, 1992). Rhodopsin was the first palmitoylated GPCR to be identified (Ovchinnikov Yu *et al.*, 1988). Owing to the hydrophobic nature of palmitoylation, it was suggested that the palmitate moieties inserted into the plasma membrane would create a fourth cytoplasmic loop, experimentally supported with fluorescent palmitate analogue studies (Moench *et al.*, 1994a; Moench *et al.*, 1994b). Since then many other GPCRs have been identified, 80% having in common the presence of one or two cysteine residues in the membraneproximal domain of the carboxy tails, but no strict consensus sequence (Ponimaskin *et al.*, 2001). Bouvier and coworkers analyzed the reversibility and stability of palmitoylation upon β-AR activation. Agonist treatment increased the turnover rate of palmitate linked to the receptor (Mouillac *et al.*, 1992; Ponimaskin *et al.*, 2001). However, agonist-stimulated palmitate turnover has not been found in all GPCRs studied. Palmitoylation was found to restrain access to the phosphorylation sites in the carboxy terminal tail responsible for receptor downregulation (Moffett *et al.*, 1996). Later, work with a novel β_2-AR/Gα_s fusion chimera demonstrated that early steps in the agonist activation process lead to the depalmitoylation of both receptor and G protein, and is temporally separated from the repalmitoylation of these proteins (Loisel *et al.*, 1999).

1.4.5.3.2 Specific Membrane Retention of Integral Membrane Proteins

Palmitoylation may serve to enhance surface expression and divert proteins from a degradative pathway, as shown for the GPCRs vasopressin V1a, V2, and A1 adenosine receptors (Sadeghi *et al.*, 1997; Gao *et al.*, 1999; Hawtin *et al.*, 2001). Inhibition of receptor-mediated endocytosis of transferrin is associated with the covalent modification of the transferrin receptor with palmitic acid (Alvarez *et al.*, 1990). The cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking, and prevents the receptor from entering lysosomes (Schweizer *et al.*, 1996).

1.4.5.4 Involvement in Protein-Protein Interactions

Most studies evaluating the importance of palmitoylation in protein-protein interactions have used heterotrimeric G proteins as a model. Palmitoylation increases the affinity of Gα_s for βγ subunits 5-fold (Iiri *et al.*, 1996). However, palmitoylation-defective yeast α subunit Gpa1p and Gα_{i1} interact with βγ similarly to WT (Song and Dohlman, 1996; Wise *et al.*, 1997). When the nicotinic acetylcholine receptor was synthesized in the presence of the acylation inhibitor, cerulenin, its five subunits failed to assemble into a functional pentamer (Olson *et al.*, 1984). Cerulenin and numerous analogues are believed to inhibit protein palmitoylation through covalent alkylation of the presumptive PAT, but have long been known to inhibit cell proliferation and fatty acid synthase to varying degrees depending on the particular analogue (Lawrence *et al.*, 1999; De Vos *et al.*, 2001).

In addition to membrane association, artificially monoacylated ribonuclease A proteins with the longest attached chains (14 and 16 carbon atoms) tended to self-associate, mediated by acyl group interactions (Roy *et al.*, 1997). This has also been shown for synthetic C-terminal lysine myristoylated insulin, where it enhances protein-protein interactions in dimer formation (Olsen and Kaarsholm, 2000). Oligomerization of GPCRs has recently been discovered; it is possible that palmitoylation assists in stabilizing the clustering of these receptors (Angers *et al.*, 2001, 2002) These observations suggest that palmitoylated proteins may facilitate the formation of oligomers inside cells.

The tetraspanin protein family includes CD9, CD37, CD53, CD63, CD81, CD82 and CD151. All contain four TMDs, incorporate [^{3}H]palmitate, and associate with various surface molecules and with each other to build a network of molecular interactions termed the tetraspanin web. The interaction of tetraspanins with each other seems to be central for the assembly of the tetraspanin web. The palmitoylation status of tetraspanins did not influence their partitioning into detergent-resistant membranes but contributed to the interaction with other tetraspanins. Mutant nonpalmitoylated tetraspanins are diffusely distributed, showed markedly diminished stability during biosynthesis, and alter cellular morphology in kidney epithelial cells (Charrin *et al.*, 2002; Yang *et al.*, 2002).

1.4.5.5 Regulation of Enzymatic Activity by Active Site Fatty Acylation

In the mitochondrial matrix, the reversible covalent attachment of C8-C18 fatty acids via a thioester linkage to certain mitochondrial proteins at their active sites may play a role in metabolic regulation in the mitochondrion (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994; Corvi *et al.*, 2001). Despite the fact that no PAT activity was identified or required for mitochondrial palmitoylation to occur, mitochondrial thioesterases are known to exist (Lee and Schulz, 1979; Gross, 1984; Svensson *et al.*, 1995; Poupon *et al.*, 1999). Thus, acylation/deacylation cycles of these proteins may be a key regulatory feature.

1.4.5.6 Palmitoylation and Protein Translocation Across Membranes

In addition to membrane association, palmitoylation of otherwise soluble proteins may cause complete translocation across lipid membranes (Schmidt, 1989). Stearoylation of trypsin and chymotrypsin resulted in the accessibility of multilamellar liposome-enclosed substrate to these enzymes (Levashov *et al.*, 1984; Levashov *et al.*, 1985). Monoacylated RNase A was able to cross an *in vitro* model of the blood-brain barrier if the acyl chain was at least 16 carbon atoms long (Chopineau *et al.*, 1998). The cutaneous absorption of multipalmitoylated IFNα was about 5-6 times greater than the parent protein (Foldvari *et al.*, 1998). Such translocation across a bilayer may be the mechanism for the localization of a family of hydrophilic acylated surface proteins (HASPs) to the extracellular face of the plasma membrane in infective parasite stages. These surface proteins lack a recognizable ER secretory signal sequence, transmembrane spanning domain, or GPI anchor consensus sequence. The N-terminal domain of HASPB contains primary structural information that directs both N-myristoylation and palmitoylation (Denny *et al.*, 2000). Palmitoylation of peptide antigens via a thioester bond dramatically increases their immunogenicity (Beekman *et al.*, 1997). This may cause membrane association and/or translocation of acylated proteins such as glutamic acid decarboxylase (GAD-65) and IL-1α. Remarkably, both proteins are the targets of autoantibodies in the autoimmune disorders diabetes mellitus type I and rapidly progressive idiopathic pulmonary fibrosis (Solimena *et al.*, 1993; Ogushi *et al.*, 2001).

1.4.5.7 Palmitoylation and Targeting to Membrane Subdomains

Brown and Rose identified the association of a GPI-anchored protein, placental alkaline phosphatase (PLAP), with glycolipid-enriched, low-density membranes, resistant to detergent solubilization at 4°C (Brown and Rose, 1992). This seminal paper laid the foundation for the concept of lipid rafts/detergent resistant membranes (DRMs) and the propensity for lipid-modified proteins to be associated with these membrane domains. Sargiacomo and coworkers were the first to report the cofractionation of N-terminally acylated proteins with a presumed marker of these microdomains, caveolin (Sargiacomo *et al.*, 1993). In Triton X-100 (TX-100) insoluble, glycolipid-enriched membrane fractions, the noncovalent association between myristoylated and palmitoylated proteins and GPI-anchored proteins such as DAF in coimmunoprecipitation experiments was initially believed to be mediated by fatty acylation (Shenoy-Scaria *et al.*, 1993). The GPI anchor of DAF and Cys3 in Lck were found to independently mediate DRM/caveolae association (Rodgers *et al.*, 1994; Shenoy-Scaria *et al.*, 1994). Later, Brown and coworkers suggested that two saturated acyl chains are needed to target a protein to DRMs, such as dipalmitoylated GAP-43 or the myristoylated and palmitoylated $G\alpha_o$ (Arni *et al.*, 1998).

It is now recognized that rather than being a vast homogenous array of phospholipids and other lipid species and proteins, the plasma membrane contains discrete microdomains, or lipid rafts, enriched in specific types of lipids and proteins. These microdomains are also referred to as DRMs (detergent resistant membranes), DIGs (detergent insoluble glycosphingolipid enriched membranes), DIMs (detergent insoluble membranes), TIFFs (TX-100 insoluble floating fraction), GEMs (glycolipid-enriched membranes), and caveolin-enriched membranes (Parton and Simons, 1995; Simons and Ikonen, 1997; Anderson, 1998; Brown and London, 1998b, a; Galbiati *et al.*, 1999; Kurzchalia and Parton, 1999; Resh, 1999). For consistency, DRMs will be used to describe domains isolated by biochemical fractionation methods, and lipid rafts will be used to describe a similar but not identical domain enriched in lipid species but not having the constraints of detergent insolubility.

These membrane subdomains have been reported to be resistant to extraction with cold, nonionic detergents, and are enriched in glycosphingolipids, free cholesterol, and saturated phospholipids (Harder and Simons, 1997; Fridriksson *et al.*, 1999). In many cases, several lipidated proteins including NRTKs, G proteins such as $G\alpha_o$ and $G\alpha_i$, GPI-anchored proteins, and caveolin are also enriched in these membranes (Simons and Ikonen, 1997; Kurzchalia and Parton, 1999; Oh and Schnitzer, 1999). DRMs have been described as liquid-ordered (l_o) phase lipid domains dispersed in a liquid disordered (l_d) lipid bilayer (Brown and London, 1998b, a). DRM phospholipids are believed to primarily contain saturated fatty acyl chains (Brown and London, 1998b). As well, detergent insolubility exists in the absence of proteins, suggesting that DRMs arise from lipid-lipid interactions. Sphingolipids, with long saturated acyl chains, have an unusually high T_m, and are believed to have a tight packing ability leading to their detergent insolubility. The l_o phase was first identified in binary mixtures of saturated PC and cholesterol, and requires cholesterol to form. This l_o phase has properties that are intermediate between those of the gel and fluid phases. Like the gel phase, the l_o phase has tight acyl-chain packing and relatively extended acyl chains. Unlike the gel phase, acyl chains in the l_o phase have rapid lateral mobility in the bilayer (Brown and London, 1997).

Membrane raft size has been shown to vary from 20 to 400 nm in diameter, with assumptions used that can lead to large errors is size estimation (Varma and Mayor, 1998; Hooper, 1999; Jacobson and Dietrich, 1999; Pralle *et al.*, 2000). A collection of data suggests that rafts are small and dynamic, but can be stabilized into caveolae or larger domains by specific proteins as well as by antibody crosslinking or patching (Harder *et al.*, 1998; Rietveld and Simons, 1998). Lipid rafts from different origins have been shown to be similar in many properties, but unique in others (*e.g.* constituent proteins or lipids), and support the existence of many kinds of lipid rafts (Parkin *et al.*, 1996; Iwabuchi *et al.*, 1998; Kurzchalia and Parton, 1999; Waugh *et al.*, 1999). The distinction between caveolae and rafts has been validated in a recent report (Sowa *et al.*, 2001). Studies involving MARCKS, Src and polybasic peptides led to the observation that polybasic protein regions may induce acidic phospholipid (PS and PIP_2) microdomain formation due to multivalent binding (>1 acidic lipid per polybasic region) (Buser *et al.*, 1995; Glaser *et al.*, 1996; Denisov *et al.*, 1998). This suggests that anionic phospholipid-enriched rafts may exist in cells, although experimental artifacts may make this possibility less tenable (Pike *et al.*, 2002).

Caveolin, a 21 kDa palmitoylated semi-integral membrane cholesterol binding protein (Murata *et al.*, 1995; Parat and Fox, 2001) has been utilized as a marker for caveolae (Song *et al.*, 1996; Song *et al.*, 1997b; Orlandi and Fishman, 1998; Oh and Schnitzer, 1999). Caveolae, 50-70 nm invaginations of the plasma membrane, are resistant to solubilization with TX-100 at 4°C and enriched in caveolin, which has been used as a marker for their purification (Anderson, 1998). These may represent a specialized DRM with a cytoplasmic coat of oligomerized caveolin molecules. Due to the comparable properties (detergent insolubility, lipid constituents, and buoyancy) between caveolae and DRMs/lipid rafts, caveolin has also been utilized as a marker of DRMs/lipid rafts during isolation. However, selective isolation procedures indicated that caveolae do not equate with detergent-insoluble lipid rafts (Hooper, 1999), and that non-caveolar lipid rafts do exist as separate entities (Iwabuchi *et al.*, 1998). Caveolin possesses a complex intracellular cycling pathway involving plasma membrane, caveolae, ER, ERGIC, Golgi, and cytosol, and as such is found in a variety of membranes (Conrad *et al.*, 1995; Uittenbogaard *et al.*, 1998).

N-terminally acylated proteins, such as NRTKs, GAP-43, Gα subunits, and ecNOS have been shown to be enriched in 4°C TX-100 insoluble structures and/or caveolae (Arreaza *et al.*, 1994; Shenoy-Scaria *et al.*, 1994; Melkonian *et al.*, 1995; Robbins *et al.*, 1995; van't Hof and Resh, 1997; Arni *et al.*, 1998; Galbiati *et al.*, 1999; Melkonian *et al.*, 1999). In those proteins, loss of acylation resulted in the loss of functional fractionation to lipid rafts. Also, kinetic studies by Resh and coworkers with NRTKs have shown that targeting to membranes occurs first, followed by a slower partitioning into detergent-resistant membranes (van't Hof and Resh, 1997). The current model of raft targeting of acylated proteins, forwarded primarily by Brown and colleagues, postulates that the biophysical preference of saturated acyl chains in acylated proteins for densely packed areas of similar saturated acyl chain phospholipids and cholesterol would drive partitioning of dually acylated proteins into lipid rafts (London and Brown, 2000). In most cases, two saturated acyl chains are believed to be required for partitioning of the proteins into l_o cholesterol and glycosphingolipid-enriched membrane microdomains (Schroeder *et al.*, 1994; Arni *et al.*, 1998; Schroeder *et al.*, 1998). In contrast, proteins modified by single acyl chains or bulky, branched hydrophobic lipids (farnesyl and geranylgeranyl) are not believed to partition favorably into l_o membrane rafts (Melkonian *et al.*, 1999; Moffett *et al.*, 2000). However, other reports show that prenyl and myristate groups alone are sufficient to confer enrichment in lipid rafts as defined by cofractionation with caveolin-enriched membranes (Song *et al.*, 1997a; Michaely *et al.*, 1999). Alternatively, protein-protein interactions with caveolin could drive fatty acylated proteins into caveolae, as numerous lipidated proteins contain a caveolin binding domain mediating association with this scaffolding protein (Li *et al.*, 1996).

The mechanism by which dually acylated proteins accumulate in lipid rafts has not been conclusively determined. For NRTKs, myristoylation is necessary but not sufficient for enrichment in rafts. Palmitoylation of NRTKs may be a critical determinant for DRM enrichment. NRTKs with a Cys3 (Fyn, Lck, and p59 Hck isoform) are palmitoylated and associate with DRMs (Rodgers *et al.*, 1994; Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995). Nonpalmitoylated NRTKs such as Src and p61 Hck isoform are not enriched in DRMs in some reports (Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995), but are in others (Song *et al.*, 1997a). Engineering a palmitoylation site into Src or p61 Hck results in palmitate incorporation and inclusion into DRMs. The palmitoylation requirement for inclusion into DRMs is also supported by studies of other dually acylated proteins, such as GAP-43 (Maekawa *et al.*, 1997; Arni *et al.*, 1998) and the Gα subunits (Song *et al.*, 1997a; Okamoto *et al.*, 1998).

As a gradient of cholesterol and sphingolipids exists in cells, ranging from low or nonexistent in ER membranes, more abundant across the Golgi stack to highly enriched in endosomes and PM, the location of lipid rafts/DRMs may be more widespread than the PM (Bretscher and Munro, 1993; Brown, 1998). Membranes of the Golgi and endocytic pathways all contain significant amounts of cholesterol and sphingolipid, and thus would be hypothesized to support a lipid raft microenvironment (Brown and London, 1998b). Thus, certain levels of cholesterol and sphingolipids, achievable in certain organelle membranes, are needed to form lipid rafts and provide a site for proteins to associate with. Reduction of cellular cholesterol via cholesterol synthesis inhibition or cholesterol extraction agents (such as filipin, nystatin, or MβCD), or inhibition of synthesis of cellular sphingolipids, can lead to disruption of DRM/rafts and the missorting or redistribution of associated proteins. Such observations lend support to the raft model by suggesting that these lipid constituents are required at certain threshold levels to form such domains, and proteins depend on their formation to associate with them (Christian *et al.*, 1997; Brown and London, 1998b; Orlandi and Fishman, 1998; Park *et al.*, 1998; Kasahara and Sanai, 1999).

A functional role for DRM/raft formation appears to be to create a clustered signal transducing microenvironment. In addition to restricting proteins from diffusing throughout the cell volume by membrane targeting, microdomain localization may restrict even further the diffusible range of signaling proteins. This increases the local protein concentration and probability of productive protein-protein interactions by favoring collision coupling of properly oriented protein moieties on the membrane surface (Bigay *et al.*, 1994; van't Hof and Resh, 1999). Rafts also function in sorting and trafficking through the secretory and endocytic pathways, especially in polarized cells, as classically described by Simon and coworkers (Keller and Simons, 1997; Simons and Ikonen, 1997; Verkade and Simons, 1997).

1.5 THESIS OBJECTIVE

The objective of the current study was to investigate the roles of myristoylation and palmitoylation, found within short sequences from cellular signal transduction proteins, in subcellular localization. Specifically, we sought to determine how different N-terminally acylated sequences can confer the observed ability to localize proteins to specific cellular membranes, rather than just a general membrane affinity.

The heterogeneous nature of N-terminal fatty acylated sequences studied includes differences in the number of palmitates and the position(s) that they occupy, in the presence/absence of myristate or positive charges, and in amino acid composition (Casey, 1995; Milligan *et al.*, 1995). In an attempt to explain or rationalize this observed heterogeneity, we postulated that these variably acylated sequences might contain specific subcellular localization information. To test this hypothesis, various acylated N-terminal sequences, corresponding to the first 11-16 amino acids of signal transducing proteins, were fused to the cytosolic reporter protein green fluorescent protein (GFP), and the fate of the resulting chimeric proteins studied using confocal microscopy. Using minimal sequences appended to the reporter protein, potential contributions of other binding domains and protein modules found in the full-length proteins that the sequences are derived from were avoided. This approach was conceived at a time when the majority of studies in the field were based on loss-of-function studies, which suffered from the inability to attribute a protein's observed subcellular localization solely to a mutation affecting the protein's acylation status. GFP was selected as reporter protein in these studies due to its intrinsic fluorescence properties and previous usage as a reporter protein for subcellular localization studies (Pines, 1995; Gerdes and Kaether, 1996; Girotti and

Banting, 1996; Liu *et al.*, 1997). Briefly, our results showed that different combinations of myristoylation, palmitoylation, and polybasic domains appended to GFP led to acylation-dependent specific subcellular localization in COS-7 cells.

Following these initial studies, we wanted to characterize the mechanism of acylation-dependent subcellular localization. We postulated that, based on a number of reports in the literature, DRMs or lipid rafts may be involved in the association of acylated chimeric GFPs with membranes. By using multiple fractionation and immunocytochemical methods, we characterized the cellular, biochemical and biophysical basis of the raft association of these N-terminally acylated GFP chimeric proteins. Since lipid rafts have been reported to be enriched in cholesterol and sphingolipid, and caveolae enriched in caveolin, we utilized novel reagents to detect the intracellular colocalization of the chimeric GFPs with these two lipid raft constituents and with caveolin, the prototypical marker for caveolar DRMs. Briefly, we showed that membranes enriched in cholesterol and sphingolipids are also enriched in dually acylated or myristoylated plus polybasic domain GFP chimeras. Paradoxically, DRMs/lipid rafts prepared by two different methods were devoid of acylated GFPs in our system.

The results of my investigations have allowed me to formulate a more complete model of N-terminal acylation-dependent protein localization. This work also provides additional insights into the mechanisms governing the spatial and temporal regulation of signal transduction cascades in cellular systems, and hopefully will contribute to the understanding of pathogenic states caused by or involving acylated proteins.

CHAPTER 2

FUNCTIONAL ROLES FOR FATTY ACYLATED NH_2-TERMINAL DOMAINS IN SUBCELLULAR LOCALIZATION

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2.1 ABSTRACT

Several membrane associating signals, including covalently linked fatty acids, are found in various combinations at the N-termini of signaling proteins. The function of these combinations was investigated by appending fatty acylated N-terminal sequences to green fluorescent protein (GFP). Myristoylated plus mono/dipalmitoylated GFP chimeras and a GFP chimera containing a myristoylated plus polybasic domain were localized similarly to the plasma membrane and endosomal vesicles, but not the nucleus. Myristoylated, nonpalmitoylated mutant chimeric GFPs were localized to intracellular membranes including endosomes and ER, and were absent from the plasma membrane, Golgi, and nucleus. Dually palmitoylated GFP was localized to the plasma membrane and Golgi region, but not detected in endosomes. Nonacylated GFP chimeras, like GFP, showed cytosolic and nuclear distribution. Our results demonstrate that myristoylation is sufficient to exclude GFP from the nucleus and associate with intracellular membranes, but plasma membrane localization requires a second signal, namely palmitoylation or a polybasic domain. The similarity in localization conferred by the various myristoylated and palmitoylated/polybasic sequences suggest that biophysical properties of acylated sequences and biological membranes are key determinants in proper membrane selection. However, dual palmitoylation in the absence of myristoylation conferred significant differences in localization, suggesting that multiple palmitoylation sites and/or enzymes may exist.

2.2 INTRODUCTION

Inside the living cell, covalent lipid modifications of proteins are utilized to alter the physical and functional properties of proteins. This process, called protein lipidation, is subdivided into four major categories based on the identity of the lipid attached to the protein. Prenylation, glypiation, cholesteroylation and fatty acylation represent covalent modifications of proteins by isoprenoids (farnesyl and geranylgeranyl), glycosylphosphatidylinositol (GPI) structures, cholesterol, and fatty acids, respectively (Casey, 1995; Porter *et al.*, 1996; Bhatnagar and Gordon, 1997). Recently, protein lipidation has been shown to be an important new type of protein modification involved in several aspects of cellular signaling (Casey, 1995; Milligan *et al.*, 1995; Mumby, 1997; Dunphy and Linder, 1998). However, the rationale for attaching various and multiple lipid moieties to proteins is still not well understood.

Protein fatty acylation involves two main types of fatty acids, myristate and palmitate (Bhatnagar and Gordon, 1997). Myristoylation is the permanent cotranslational linkage of the 14-carbon fatty acid myristate to an N-terminal glycine of a protein via an amide bond catalyzed by the enzyme *N*-myristoyl transferase (NMT). Palmitoylation is the reversible post-translational linkage of the 16-carbon fatty acid palmitate to variably located cysteine residues via a thioester bond. Membrane-associated protein *S*-acyltransferase (PAT) activities have been partially purified (Berthiaume and Resh, 1995; Dunphy *et al.*, 1996; Veit *et al.*, 1996a; Veit *et al.*, 1998), and a cytosolic acyl protein thioesterase (APT1) has recently been cloned (Duncan and Gilman, 1998), but their relevance to fatty acylation regulation remains to be elucidated.

Examples of proteins having both myristate and palmitate at their N-termini include several Src-related protein tyrosine kinases (PTKs) (Alland *et al.*, 1994; Resh, 1994), α subunits of heterotrimeric G proteins (Wedegaertner *et al.*, 1995; Morales *et al.*, 1998; Galbiati *et al.*, 1999), and A-kinase anchoring protein AKAP18 (Fraser *et al.*, 1998). In addition, several proteins contain myristate and a polybasic domain, including Src and myristoylated alanine-rich C-kinase substrate (MARCKS) (Kaplan *et al.*, 1992; McLaughlin and Aderem, 1995). Some contain two or more covalently linked palmitates at their N-termini, as in GAP-43/neuromodulin or L-type voltage-dependent calcium channel subunit β_{2a} (Zuber *et al.*, 1989; Chien *et al.*, 1998).

N-myristoylation, like farnesylation, has been shown to be insufficient by itself to stably anchor proteins to membranes (Shahinian and Silvius, 1995). Typically, myristoylation and prenylation signals are linked to a second signal that assists in membrane anchoring. One secondary signal is a series of positively charged residues adjacent or distal to the lipidation site, a combination utilized by proteins such as c-Src, K-Ras and MARCKS (Hancock *et al.*, 1990; Resh, 1993; McLaughlin and Aderem, 1995). Another has been shown to be palmitoylation, utilized by Yes, Fyn and Lck PTKs and H- and N-Ras GTPases. Furthermore, in those PTKs and GTPases, myristoylation and farnesylation have been shown to be prerequisites for palmitoylation to occur (Koegl *et al.*, 1994). The presence of a polybasic domain second signal in the case of multiply palmitoylated proteins like GAP-43 has been proposed but remains controversial, as conflicting reports of its significance exist (Liu *et al.*, 1993, 1994).

The heterogeneous nature of N-terminal fatty acylated sequences includes differences in the number of palmitates and the position(s) that they occupy, in the presence/absence of myristate or positive charges, and in amino acid composition (Casey, 1995; Milligan *et al.*, 1995). In an attempt to explain or rationalize this observed heterogeneity, we postulated that these sequences might contain specific subcellular localization information. To test this hypothesis, various N-terminal sequences corresponding to the first 11-16 amino acids of PTKs, Gα proteins and GAP-43 were fused to the cytosolic reporter protein green fluorescent protein (GFP), and the fate of the resulting chimeric proteins was studied using confocal microscopy. GFP has been selected as reporter protein in our studies due to its intrinsic fluorescence properties and since it has previously been demonstrated to be an excellent reporter protein for subcellular localization studies (Pines, 1995; Gerdes and Kaether, 1996; Girotti and Banting, 1996; Liu *et al.*, 1997). Our data support the possibility of more than one role for the various fatty acylated N-terminal domains in subcellular localization.

2.3 MATERIALS AND METHODS

2.3.1 Plasmid Design and Construction

Appropriate oligonucleotides containing *Bgl*II and *Bam*HI restriction endonuclease recognition sites at the 5′ and 3′ ends, respectively, S65T GFP cDNA, and Vent DNA polymerase (New England Biolabs) were used in the PCR reactions. Amplified fragments were digested with corresponding restriction enzymes, gel purified, and ligated into the appropriately digested pCMV5 mammalian expression vector (Andersson *et al.*, 1989). pCMV5 possesses the SV40 replication origin and uses the strong cytomegalovirus promoter to drive efficient heterologous gene expression in SV40 large T antigen transformed cells, such as COS-7 cells. The plasmid also contains the human growth hormone fragment containing transcription termination and polyadenylation signals for correct eukaryotic mRNA processing. Large scale plasmid preparations were done for each construct using the Monster 4G Maxiprep Kit (Bio-101) and each construct was sequenced using automated DNA sequencing on Applied Biosystems Sequencer in the Biochemistry DNA core facility (University of Alberta) and shown to conform to the original design. Standard molecular biology protocols were utilized (Sambrook *et al.*, 1989). Further details regarding these items may be found in the **Appendices 6.1-6.6**.

2.3.2 Cell Lines, Antibodies, and Reagents

COS-7 cells were from the American Type Culture Collection (USA), and were maintained in 10% fetal bovine serum in Dulbecco's Modified Eagle's Medium (Gibco BRL) with 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate plus 2 mM L-glutamine (Sigma), and passed twice per week using a 0.25% trypsin/1 mM EDTA wash (Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

To prepare frozen stocks, sufficient trypsin-EDTA was added to each confluent 100 mm dish to cover the monolayer, then aspirated. Dishes were incubated at 37°C until cell sloughing began; 1 ml freezing media (70% DMEM, 20% FBS, 10% dimethylsulfoxide) was added to resuspend the cells and the entire volume was aliquoted into a cryovial (Wheaton) and immediately frozen at –80°C. To thaw cells, vials were rapidly thawed in a

37°C waterbath, and contents were transferred to a 100 mm dish containing maintenance medium. Two passages were allowed before cells were utilized in experiments.

Rabbit polyclonal anti-GFP antibody (Ab) was developed in our laboratory using highly purified recombinant GFP made in *E. coli* as antigen; goat polyclonal anti-calreticulin Ab was a kind gift from Dr. M. Michalak (University of Alberta, Edmonton, Alberta, Canada); mouse anti-GFP was from Chemicon; rabbit anti-giantin was from Dr. E.K. Chan (Scripps Institute, La Jolla, U.S.A.); mouse monoclonal anti-CD63 was from Dr. K. Jimbo (University of Alberta). Mouse monoclonal anti-Yes PTK was from Transduction Laboratories. Donkey anti-rabbit IgG-Texas Red (TR) and IgG-FITC, donkey anti-mouse IgG-TR and IgG-FITC, and donkey anti-goat IgG-TR and IgG-FITC secondary Abs, and normal donkey serum (NDS) were obtained from Jackson ImmunoResearch Laboratories. Protein A sepharose CL-4B was from Amersham-Pharmacia. BODIPY TR-ceramide (stored as a DMSO stock), and DiI were obtained from Molecular Probes. Paraformaldehyde, nocodazole and TX-100 were from Sigma. DiI-LDL was prepared and stored in 0.9% NaCl and 0.01% EDTA as described (Pitas *et al.*, 1981). Nocodazole (NZ) was stored as a 4 mM stock in DMSO at -20°C.

2.3.3 Transfection, Live Cell Fluorescence, and Immunofluorescence Microscopy

COS-7 cells were seeded at 1.5 x 10^5 cells/well into 6-well tissue culture plates containing flame-sterilized 22 X 22 mm glass coverslips (No. 1 thickness, Fisher Scientific) coated with 20 µg/ml poly-L-lysine solution (Sigma) to promote cell adhesion. Poly-L-lysine was diluted in water and added to coverslips for five minutes at room temperature with agitation. Coverslips were then rinsed in distilled water and allowed to dry for at least 2 h before use. 24 h after seeding, cells grown on coverslips were transfected for 2.5 h using the DEAE-Dextran/DMSO shock method (Cullen, 1987).

10 mg/ml (25X) working solutions of DEAE (diethylaminoethyl)-dextran (chloride form, 500 000 MW; Sigma) were prepared by slowly dissolving the dextran in sterile PBS (phosphate buffered saline pH 7.4; 2.68 mM potassium chloride, 1.47 mM potassium dihydrogen phosphate, 137 mM sodium chloride, 4.27 mM sodium phosphate) heated to 80°C. Once dissolved, the solution was heated to boiling using a microwave, then stored at 4°C. Chloroquine (diphosphate salt; Sigma) was prepared as a 100 mM stock in sterile PBS and stored under tinfoil at -20°C. 1 µg/ml working solutions of each N-terminal sequence-GFP construct were prepared from maxi-prep DNA in sterile TE and sterile filtered through a Spin-X tube (Costar). DMSO (dimethylsulfoxide; Caledon) was prepared as a 10% solution in warm sterile PBS just before use.

COS-7 cells below cell passage 16 were utilized for transfection. Cells to be transfected are seeded to 6-well plates twenty-four hours before transfection. 50% confluent 6-well dishes of COS-7 cells were washed twice in PBS and overlaid with 400 µl of transfection mix per dish. The transfection mix consists of 1 ml DMEM supplemented with 10% NuSerum (Becton-Dickinson), 40 µl of 25X (10 mg/ml) DEAE-dextran (400 µg/ml final), 1 µl of 100 mM chloroquine (0.1 mM final) and 1.25 µg of construct DNA. The transfection mix was left on the cells for 2.5 hours at 37°C, 5% CO_2, then aspirated before 4 ml of 10 % DMSO was added to each dish for two minutes at room temperature. Cells were washed twice in PBS and 1 ml maintenance medium (DMEM plus FBS) was added. Cells were allowed to express chimeric GFP proteins for 16-24 hours post-transfection before use in experiments in order to correlate observable cellular fluorescence with protein expression.

Cells were analyzed 16-24 h after transfection to allow for GFP chromophore development (Olson *et al.*, 1995). For live cell fluorescence analysis, coverslips were removed from media, washed with pre-warmed PBS and mounted on glass slides (Corning) in PBS using vacuum grease or nail polish as a sealant. For immunocytochemistry, the methods described below are modifications of those found in Harlow and Lane (Harlow and Lane, 1988). For cell fixation, 4% paraformaldehyde pH 7.4 (Sigma) was prepared by adding paraformaldehyde to distilled water while stirring, then heating to 60°C. 1 N NaOH was added dropwise while swirling until the white precipitate dissolved. The solution was cooled to room temperature and 10X PBS was added to a final concentration of 1X. The solution was prepared just before use. Cells were washed in PBS, fixed in 4% paraformaldehyde in PBS pH 7.4 for 20 min, and permeabilized with 0.1% TX-100 in PBS for 1 min at room temperature. After permeabilization, coverslips were washed twice in PBS and 80 µl of blocking solution was dripped onto each coverslip and incubated for one hour at 37°C, 5% CO_2. All subsequent incubations were carried out at 37°C, 5% CO_2. Blocking solution was replaced with 80 µl of primary antibody solution and incubated for one hour; coverslips were washed four times over five minutes with PBS and 80 µl of appropriate secondary antibody (FITC or TR-conjugated) was applied for one hour. Coverslips were mounted after washing four times over five minutes with PBS.

All antibodies used were diluted in 4% NDS in PBS to prevent nonspecific binding. For colocalization of GFP chimeras and various organelles, the intrinsic GFP fluorescence, mouse anti-GFP (1:200), or rabbit anti-GFP (1:2000) was used, and anti-calreticulin (1:50), anti-giantin (1:2000), or anti-CD63 (1:100) was used to detect the ER, Golgi, and lysosomes, respectively. BODIPY TR-ceramide (1.5 µM) was added for 0.5-2 h before viewing living transfected cells to detect the Golgi apparatus, as reported (Ralston, 1993).

DiI-LDL (1 μg/ml) was added to living transfected cells 1 h prior to fixation to allow for incorporation of the fluorescent lipoprotein particle into endosomes. The red DiI-LDL fluorescence was detected with a Texas Red filter set. To disperse intracellular organelles, transfected cells were treated with 20 μM NZ for 1 h prior to fixation to allow for effective depolymerization of microtubules (Kalcheva *et al.*, 1998).

The subcellular localization of proteins was assessed by generating images acquired on two confocal laser scanning systems. The first system, used for live cell analysis, consists of a Leitz Aristoplan Fluorescence Microscope illuminated by a 100 W mercury burner for direct observation and an Ar/Kr laser with major emissions at 488, 568, and 647 nm for scanning, and 100X 1.32 NA or 63X 1.40 NA oil immersion objectives. Immunofluorescence images were obtained from coverslips mounted on glass slides using Prolong Antifade reagent (Molecular Probes) and stored in the dark at room temperature until viewed. Images were also collected with a Zeiss Laser Scanning Confocal Microscope (model LSM 510) mounted on a Zeiss Axiovert M100 inverted microscope with a 63X Apochromatic lens (1.40 NA) (Cross Cancer Institute, University of Alberta). Each image is collected within the linear range of fluorescence intensity based upon the imaging software, using FITC or Texas Red filters. Image overlays represent samples acquired using the sequential mode for double-label collection to avoid cross-talk between the fluorophores. Scans were optimized for chromophore detection. Final image manipulations were done in Adobe Photoshop 5.0. To ensure optimal comparisons, images of cells of similar sizes (15-30 μm diameter) were captured using similar pinhole and laser intensities.

2.3.4 Metabolic Labeling

Transiently transfected COS-7 cells were grown in 100 mm diameter dishes for 24 h. The following day, cells were split into two 100 mm dishes, and metabolically labeled 24 h later. For ^{3}H-myristate labeling, cells were starved of fatty acids for 1 h with 3 ml of DMEM, 2 mM L-glutamine, and 10 μg/ml BSA, to which 100 μCi per plate of [^{3}H-9,10(*n*)]-myristic acid (52.0 Ci/mmol) (Amersham-Pharmacia) was added for 4 h at 37°C. Iodopalmitic acid (a gift of Dr. M. Resh, Sloan-Kettering Cancer Center, New York, NY) was dissolved in acetone to produce a 10 mM stock and stored at -20°C. 16-[^{125}I]iodohexadecanoic acid ([^{125}I]-IC16; specific activity of 2-3 Ci/mmol), was prepared as described (Berthiaume *et al.*, 1995), without the HPLC purification step, and was used to assess palmitoylation (please see **Appendix 6.7**). For [^{125}I]-IC16 labeling, cells were starved of fatty acids for 1 h in 3 ml depletion medium DMEM containing 2 mM L-glutamine and 10 μg/ml BSA, and labeled with 90 μCi [^{125}I]-IC16 per plate for 4 h at 37°C (Alland *et al.*, 1994).

2.3.5 Immunoprecipitation

Metabolically labeled cells were rinsed twice with cold STE (salt-Tris-EDTA) buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA). Cells were scraped off the plate with a Costar cell lifter, collected by centrifugation and lysed in 2 mL of cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM $MgCl_2$, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.1 mM PMSF) for 30 min on a Nutator rotating device at 4°C. Lysates were clarified at 13,200 x *g* for 15 min in an Eppendorf microfuge to remove the nuclei and cellular debris. One ml of clarified supernatant was transferred to a clean 1.5 ml screw-cap Eppendorf tube, and incubated with either 2 µl of polyclonal anti-GFP or 2 µl of preimmune serum for 1 h at 4°C. The immune complexes were precipitated with 20 µl of 50% (w/v) slurry of Protein A-Sepharose CL-4B in cold lysis buffer for 1 h at 4°C. Samples were pelleted and washed three times with cold lysis buffer, resuspended in 20 µl sample buffer containing 20 mM DTT, boiled for 2 min, and analyzed immediately by SDS-PAGE (1.5 mm thickness, 12.5%) in Tris-glycine buffer (25 mM Tris, 225 mM glycine, 0.1 % SDS) after cooling on ice and pelleting the Protein A-Sepharose CL-4B beads at 600 x *g* for two minutes.

All methods utilized were modifications of those presented in Sambrook *et al.* (Sambrook *et al.*, 1989). Gels containing samples labeled with [^{3}H]-myristate and [^{125}I]-IC16 fatty acid were transferred onto Immobilon-P PVDF membranes (Millipore). Western blot analyses (Towbin *et al.*, 1979; Burnette, 1981) were performed by transferring separated proteins onto polyvinylidene difluoride (PVDF) membrane (Millipore) for five hours at 100 V in Tris-glycine-methanol buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8) cooled to -17°C. The transfer sandwich consisted of sponges, Whatman thick chromatography paper, polyacrylamide gel and PVDF membrane arranged within a BioRad transfer cassette. Before incorporation into the sandwich, the sponges and chromatography paper were thoroughly soaked in transfer buffer, and the PVDF membrane wetted in methanol just before overlay onto the gel.

After transfer, membranes were stained in Ponceau S (3% Ponceau S (w/v), 30% trichloracetic acid (w/v), and 30% sulfosalicylic acid (w/v)) to visualize transferred proteins. Membranes were rinsed in distilled water and blocked for one hour at room temperature, with agitation, in Blotto/5% milk blocking solution (14 mM NaCl, 2 mM Tris, 0.15% HCl (v/v), pH 7.6, with 5% powdered skim milk (w/v) added). Blocking solution was replaced with 50 ml of primary antibody solution (1:5000 dilution of rabbit polyclonal anti-GFP H184 antibody in blocking solution) and incubated for two hours at room temperature with agitation. Membranes were washed three times with PBS, 10 min per wash, and overlaid with 50 ml secondary antibody solution (1:5000 dilution of donkey anti-rabbit-horseradish peroxidase conjugate (Amersham) in blocking solution) for one hour at room temperature with agitation. Enhanced chemiluminescence (ECL) detection was performed with the ECL Plus kit (Pharmacia Biotech). Following this, PVDF membranes were put into LE Transcreen cassettes with Biomax MS film and exposed for 14 d at -80°C using for [^{3}H]-myristate samples, or into a phosphorimager screen cassette for [^{125}I]-labeled samples for 7 d (Molecular Dynamics).

2.3.6 Subcellular Fractionation

COS-7 cells expressing chimeric GFP constructs were fractionated into soluble (S100) and membrane (P100) fractions as described (Alland *et al.*, 1994), with the exception that sodium vanadate was replaced with PMSF. For every chimeric GFP construct, transfected COS-7 cells from four 100 mm dishes were subject to fractionation and immunoprecipitation. Two other modifications to the protocol were made. A total (T) fraction, representing half of the homogenate volume (approximately 1.2 ml), was aliquoted from the homogenate before centrifugation. Centrifugation of the remainder (approximately 1.2 ml) was carried out for one hour at 4°C and 100,000 x *g* in a Beckman TL100 Ultracentrifuge, using polycarbonate thick wall tubes (3.2 ml capacity) and the TLA 100.4 rotor. The supernatant (S100) represented the soluble fraction and the pellet (P100) represented the particulate fraction. The pellets were resuspended in the S100 volume of hypotonic lysis buffer (plus protease inhibitors and sucrose/EDTA) and homogenized with 5-10 strokes using a Dounce homogenizer with a tight-fitting pestle. All tubes were then adjusted to 1 ml with 5X CLB and further solubilized for one hour at 4°C with rocking. Fractions were immunoprecipitated as above and chimeras detected by Western Blot using the rabbit polyclonal anti-GFP Ab.

2.4 RESULTS

2.4.1 Plasmid Design and Construction

In order to test whether various short N-terminal fatty acylated sequences contain different subcellular targeting information, PCR-mediated mutagenesis was used (Horton *et al.*, 1989) to append various 11-16 amino acid N-terminal sequences known to be acylated from the Src PTK and Gα protein families and GAP-43 onto the cDNA of the S65T mutant of GFP (Clontech) (Heim *et al.*, 199[illegible] (**Table 2.1**). The rationale was to allow the study of features specific to the N-terminal fatty acylation domain that are important for membrane localization independent of other protein-protein interaction modules found within these signaling proteins (*e.g.* SH2 and SH3 domains of Src-related PTKs). To further investigate the specific contributions of myristate and palmitate in Fyn- and Yes-GFP, and of palmitoylation in GAP-43GFP, two series of mutations were created. One series abolished palmitoylation by mutating palmitoylated cysteine residues to serine residues (FynC3,6S, YesC3S, and GAP-43C3,4S). The other series (FynG2A and YesG2A) abolished both myristoylation and palmitoylation by substituting the glycine residue essential for myristoylation for an alanine (Gordon *et al.*, 1991). Since N-myristoylation is a prerequisite for palmitoylation of adjacent cysteine residues, prevention of myristoylation abolishes palmitoylation of those cysteines (Alland *et al.*, 1994; Resh, 1994).

To ensure proper access to the fatty acylation enzymes and potentially biological membranes, a seven amino acid hydrophilic linker (TKLTEER) corresponding to residues 12-18 of Fyn PTK was introduced between GFP and the sequence of interest. Previous reports have shown that putatively acylated N-terminal sequences appended to GFP without a linker region can prevent acylation (Liu *et al.*, 1997; Fraser *et al.*, 1998). For the Src-GFP constructs (Src_{14}GFP and Src_{16}GFP), 14 and 16 amino acids, respectively, were appended to GFP to include positively charged basic residues known to be important in membrane binding (Silverman and Resh, 1992; Sigal *et al.*, 1994).

2.4.2 Metabolic Labeling and Immunoprecipitation of Chimeric GFPs Expressed in COS-7 Cells

COS-7 cells were transiently transfected with various pCMV5 plasmids containing different chimeric GFP cDNAs and metabolically labeled. To assess proper chimera palmitoylation and myristoylation, a [^{125}I]-IC16 palmitate analog and [^{3}H-9,10(*n*)]-

Table 2.1: Fatty Acylated Sequences Appended to the N-terminus of GFP.

Protein	Amino Acid Sequence [a]	Charge[b]	First Signal[c]	Second Signal[d]	Localization[e]
	1 5 10 15				
Fyn	(M) **GC**VQ**C**KDKEA	0	myr	pal (Cys3,6)	pm + endo
Fyn(C3,6S)	(M) **G**SVQSKDKEA	0	myr	none	endo + ER
Fyn(G2A)	(M) ACVQCKDKEA	0	none	none	cyto
Yes	(M) **GC**IKSKENKS	+2	myr	pal (Cys3)	pm + endo
Yes(C3S)	(M) **G**SIKSKENKS	+2	myr	none	endo + ER
Yes(G2A)	(M) ACIKSKENKS	+2	none	none	cyto
$G\alpha_o$	(M) **GC**TLSAEERA	-1	myr	pal (Cys3)	pm + endo
Lck	(M) **GC**G**C**SSHPED	-2	myr	pal (Cys3,5)	pm + endo
Src$_{14}$	(M) **G**SSKSKPKDPSQR	+3	myr	polybasic	pm, endo, ER
Src$_{16}$	(M) **G**SSKSKPKDPSQRRR	+5	myr	polybasic	pm + endo
GAP-43	ML**CC**MRRTKQV	+3	pal	(polybasic)[f]	pm + Golgi
GAP-43(C3,4S)	MLSSMRRTKQV	+3	none	(polybasic)	cyto

[a] A neutrally charged seven amino acid linker corresponding to amino acids 12-18 of Fyn (TKLTEER) was appended between the N-terminal amino acid sequences and GFP. (M): methionine removed by methionyl aminopeptidase (cotranslational). **G**: myristoylated glycine residue (cotranslational). **C**: palmitoylated cysteine residue (posttranslational).

[b] Net charge of sequence at physiologic pH.

[c] First membrane targeting signal. myr: myristoylation; pal, palmitoylation.

[d] Second membrane-targeting signal. polybasic, a positively charged amino acid sequence.

[e] Subcellular localization of chimeric proteins as displayed in **Figs. 2.9 – 2.11**; cyto, cytosol; endo, endosomes; ER, endoplasmic reticulum; pm, plasma membrane.

[f] Putative second signal.

myristate were used. To assess protein production, Western Blot analysis was utilized.

$[^{125}I]$-IC16 labeling (**Figure 2.1A**) was evident in constructs bearing an N-terminal cysteine residue available for acylation (Fyn-, Yes-, Lck-, $G\alpha_o$-, and GAP-43GFP). Substitution of cysteine residues known to be palmitoylated for serine residues abolished incorporation of the $[^{125}I]$-IC16 label into corresponding chimeric GFPs. Mutants bearing the G2A mutation demonstrated a lack of incorporation of both $[^{125}I]$-IC16 and $[^3H]$-myristate. As this mutation is known to prevent myristoylation, which is a prerequisite for palmitoylation, these results conform with previous observations by others using full length signaling proteins (Zuber *et al.*, 1989; Parenti *et al.*, 1993; Alland *et al.*, 1994; Koegl *et al.*, 1994). Differences in labeling intensity in mono- versus dipalmitoylated chimeras could not be discerned in our system. Of note, two Src constructs, Src_{14}GFP and Src_{16}GFP, chimeras bearing no cysteines available for palmitoylation, showed very weak ^{125}I incorporation. This is probably due to metabolic interconversion of $[^{125}I]$-IC16 to $[^{125}I]$-IC14 by β-oxidation. This phenomenon has been previously observed (Alland *et al.*, 1994). $[^3H]$-myristate (**Figure 2.1B**) was incorporated into all constructs bearing a glycine residue in position 2. $[^3H]$-myristate was not incorporated into GAP-43GFP, suggesting an apparent lack of conversion from $[^3H]$-myristate to palmitate, or incorporation of $[^3H]$-myristate into palmitoylation sites by a nonspecific PAT. Finally, typical expression levels of the different GFP chimeras achieved under our experimental conditions are within an order of magnitude from one another as judged by signal intensity in our Western Blot analyses (**Figure 2.1C**). Expression levels did not appear to correlate with acylation status of GFP chimeras. Our results confirm that short (11-16 amino acid) N-terminal peptide sequences are necessary and sufficient to confer proper fatty acylation.

2.4.3 Subcellular Fractionation of Transfected COS-7 Cells

Distributions of chimeric GFPs between total, soluble and membrane fractions can be seen in **Figure 2.2**. Cells were harvested at the same time point (18-24 h post-transfection) as the live cell images in **Figure 2.3**. Myristoylated and palmitoylated or dually palmitoylated GFP constructs (Fyn-, Yes-, Lck-, $G\alpha_o$-, and GAP-43GFP) were associated with the membrane (P100) fraction to greater than 90%. Nonacylated mutants (Fyn(G2A)-, Yes(G2A)-, and GAP-43(C3,4S)GFP) and GFP alone were found nearly completely in the soluble fraction (greater than 90%). Myristoylated but not palmitoylated Fyn(C3,6S)GFP and Yes(C3S)GFPs were found distributed in both fractions, but the majority (at least ~70% on average) was found in the particulate fraction. Src_{14}GFP (net

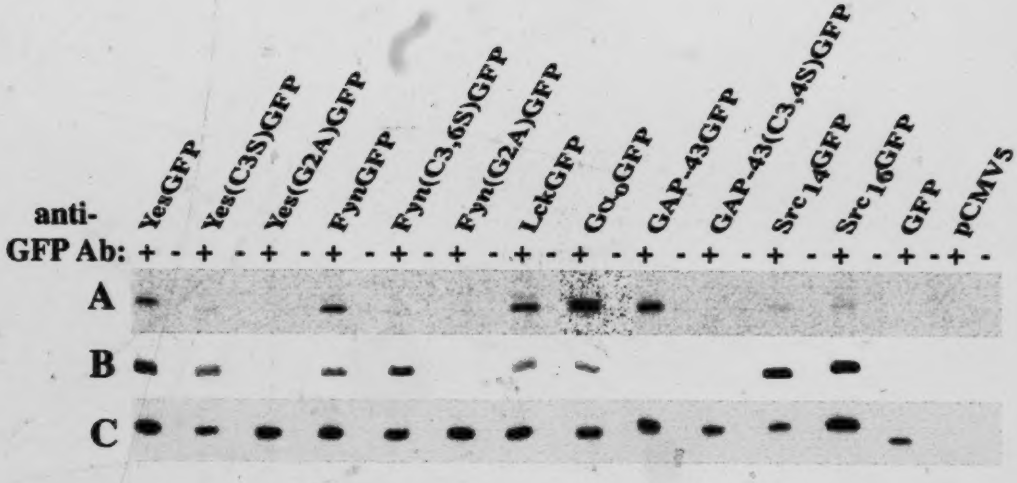
anti-GFP Ab:
YesGFP
Yes(C3S)GFP
Yes(G2A)GFP
FynGFP
Fyn(C3,6S)GFP
Fyn(G2A)GFP
LckGFP
GαoGFP
GAP-43GFP
GAP-43(C3,4S)GFP
Src14GFP
Src16GFP
GFP
PCMV5
+ - + - + - + - + - + - + - + - + - + - + - + - + - + -
A
B
C

Figure 2.1. Metabolic labeling of chimeric GFPs. COS-7 cells transiently expressing N-terminal fatty acylated GFP chimeras and mutants were metabolically labeled with ^{125}I-IC16 to assess chimera palmitoylation, (A), and with [^{3}H-9,10(*n*)]-myristic acid to assess chimera myristoylation, (B). Western blot analysis of corresponding transiently transfected COS-7 cells to assess protein production, (C). Plus (+) and minus (-) signs refer to anti-GFP serum and pre-immune serum, respectively.

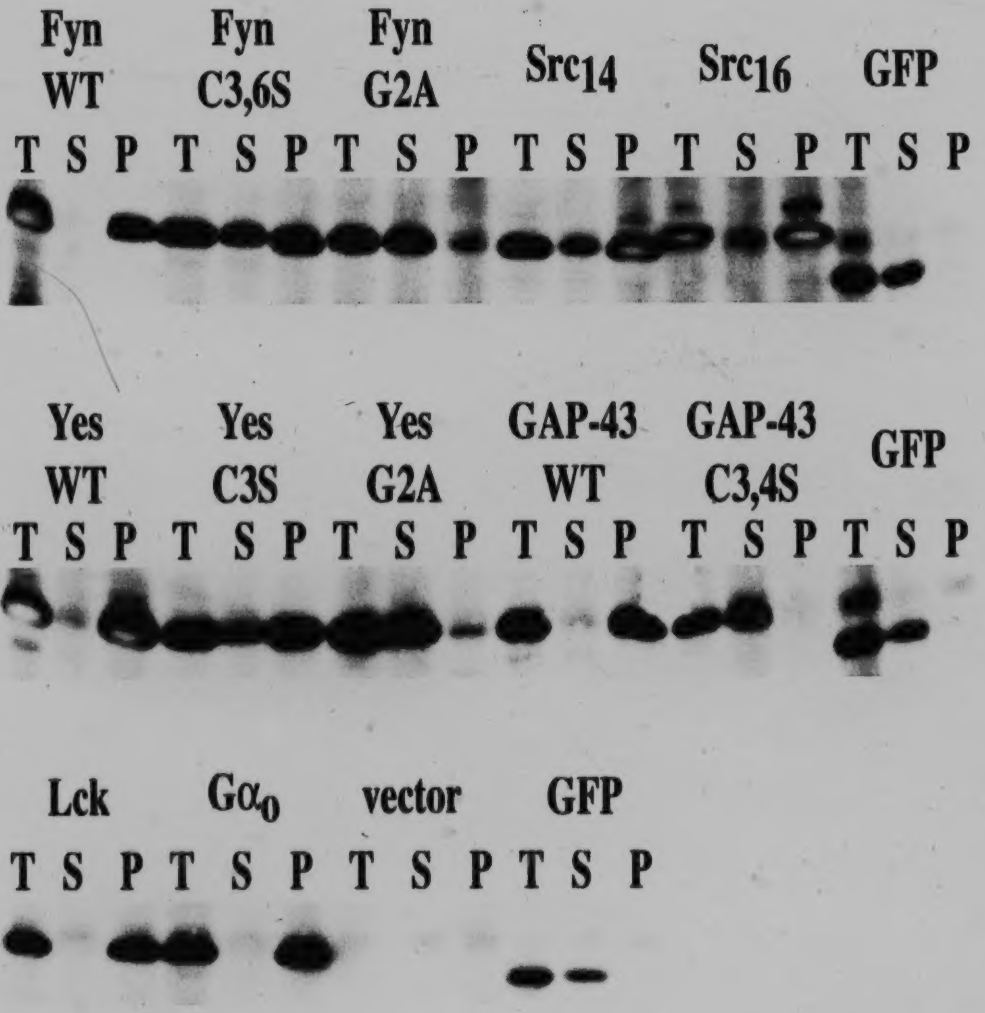
Fyn WT
Fyn C3,6S
Fyn G2A
Src14
Src16
GFP
T S P T S P T S P T S P T S P T S P
Yes WT
Yes C3S
Yes G2A
GAP-43 WT
GAP-43 C3,4S
GFP
T S P T S P T S P T S P T S P T S P
Lck
Gαo
vector
GFP
T S P T S P T S P T S P

Figure 2.2. Subcellular fractionation of COS-7 cells expressing various GFP chimeras. T, total cell lysate; S, supernatant fraction (S100); P, pellet fraction (P100).

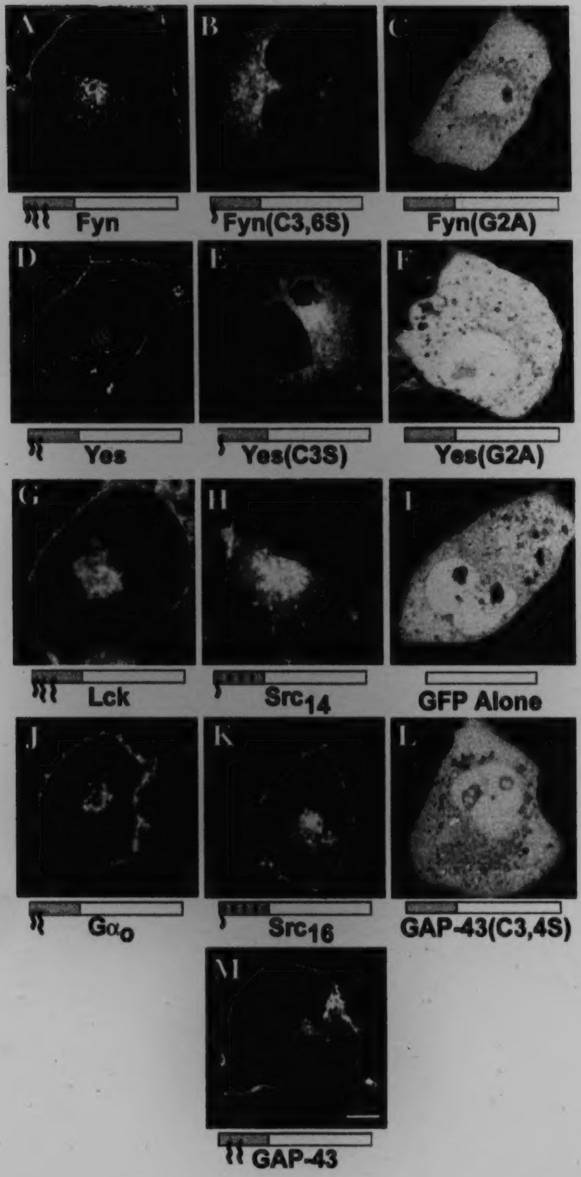
A
B
C
Fyn
Fyn(C3,6S)
Fyn(G2A)
D
E
F
Yes
Yes(C3S)
Yes(G2A)
G
H
I
Lck
Src14
GFP Alone
J
K
L
Gαo
Src16
GAP-43(C3,4S)
M
GAP-43

Figure 2.3. Subcellular localization of various N-terminal fatty acylated GFP chimeras and mutants in living COS-7 cells. GFP fluorescence of COS-7 cells expressing: (A), FynGFP. (B), Fyn(C3,6S)GFP. (C), Fyn(G2A)GFP. (D), YesGFP. (E), Yes(C3S)GFP. (F), Yes(G2A)GFP. (G), LckGFP. (H), Src_{14}GFP. (I), GFP alone. (J), $G\alpha_o$GFP. (K), Src_{16}GFP. (L), GAP43(C3,4S)GFP. (M), GAP-43GFP. Intrinsic GFP fluorescence was detected by confocal laser scanning microscopy. In the models below the images, the dark grey box: appended acylation amino acid sequence; light grey box, GFP; small acyl chain (〰), myristate; large acyl chain (〰), palmitate; plus (+) signs, polybasic region. Bar, 10 μm.

charge +3) was in the S100 fraction such that it showed an intermediate distribution between myristoylated alone constructs and Src_{16}GFP (net charge +5), which was distributed similar to that of dually acylated chimeras (vast majority in P100). In some cases, recovery yields of GFP from the cell fractionation experiments were not always 100%. This may represent losses due to nonacylated chimeras or GFP being trapped in the nucleus. Alternatively, acylated GFP chimeras could remain associated with nuclear pellet membranes. In addition, non-specific losses may occur during processing in the Dounce homogenizer, as acylated GFPs are typically "sticky" due to their hydrophobic anchors. On the other hand, in several cases, recovery was apparently 100% or very close to 100% (*e.g.* YesGFP, Src_{14}GFP and Src_{16}GFP).

Our procedure utilized to immunoprecipitate and detect GFP chimeras is extremely efficient and typically 100 ng to 1 μg of apparent protein can be recovered in a single lane on the PVDF membrane. In some lanes of **Figure 2.2**, we obtained some white areas within a band. We believe these white areas are due to the high sensitivity of ECL detection utilized combined with the large amount of GFP present on the PVDF membrane and not due to overexposure of the autoradiographic film which was typically less than 5 seconds. Those white areas within bands might represent ECL substrate depletion in the middle of the band area.

Overall, the relative distributions of these chimeric constructs correlate with the number of membrane binding signals (*e.g.* myristate, palmitate or polybasic domain) present in their N-termini. Our subcellular fractionation data also correlate with those previously reported using full-length acylated signaling proteins and chimeras (Kaplan *et al.*, 1992; Alland *et al.*, 1994; Wolven *et al.*, 1997; Arni *et al.*, 1998; Galbiati *et al.*, 1999).

2.4.4 Localization of GFP Chimeras by Confocal Microscopy

To assess whether various N-terminal fatty acylated sequences contain different subcellular localization information in cells without possible artifacts from fixation and permeabilization, we transfected and viewed living COS-7 cells expressing different chimeric GFPs (shown in **Table 2.1**). COS-7 cells expressing myristoylated and dipalmitoylated FynGFP (**Figure 2.3A**) demonstrated plasma membrane and focal perinuclear localization with nuclear exclusion and low cytosolic levels at early time points in the transient transfection procedure (*i.e.* less than 24 h post transfection). Myristoylated Fyn(C3,6S)GFP (**Figure 2.3B**), bearing a single lipid modification, was localized to multiple intracellular membranes including the nuclear envelope, and appeared enriched in perinuclear vesicles. This construct was also excluded from the nucleus, and was not detected in the plasma membrane. Finally, Fyn(G2A)GFP, a nonacylated mutant, showed widespread cytoplasmic distribution in cells with negative staining of nucleoli and vesicular/vacuolar structures (**Figure 2.3C**).

To assess whether the N-terminal combination of one myristate and one palmitate would confer localization different to a combination of one myristate and two palmitates found in FynGFP, we expressed the myristoylated and monopalmitoylated YesGFP chimera in COS-7 cells and looked for cellular distribution of the green fluorescence. Like FynGFP, YesGFP was found at the plasma membrane and perinuclear area, was excluded from the nucleus and showed low cytosolic levels (**Figure 2.3D**). Myristoylated Yes(C3S)GFP, like Fyn(C3,6S)GFP, was localized to several intracellular membranes (and also appeared enriched in perinuclear vesicles), was excluded from the nucleus, and was typically absent from the plasma membrane (**Figure 2.3E**). Nonacylated Yes(G2A)GFP showed a widespread distribution in cells (**Figure 2.3F**). Using our experimental conditions, the fluorescence patterns of dually acylated Fyn- and YesGFP chimeras were indistinguishable, as were the fluorescence patterns of corresponding myristoylated but not palmitoylated mutant chimeric GFPs.

To assess whether the amino acid composition found at the N-terminus and if the number and position of palmitates were important factors in subcellular distribution of dually fatty acylated chimeric GFPs, the myristoylated and dipalmitoylated (at positions 3 and 5) LckGFP chimera and the myristoylated and monopalmitoylated (at position 3) Gα_0GFP were expressed in COS-7 cells (**Figure 2.3, G and J**). LckGFP and Gα_0GFP were localized in a way that was apparently indistinguishable from that of FynGFP and YesGFP chimeras. As such, it appears that the amino acid composition surrounding the fatty acylated residues and the number and position of covalently linked palmitate(s) are not essential determinants for subcellular distribution in our system. The minimal feature common to all four constructs is a myristoylated glycine (in position 2) adjacent to a palmitoylated cysteine (in position 3). This dually fatty acylated motif alone may account for the similar localization observed.

In order to compare the subcellular localization information encoded by the N-terminal combination of myristate and palmitate to that of myristate and a polybasic domain, two SrcGFP constructs were engineered and expressed in COS-7 cells. Src$_{14}$GFP, bearing a myristate group and a positively charged domain bearing a net charge of +3, showed localization to the plasma membrane, nuclear membrane, and several widespread intracellular membranes/structures, and was also excluded from the nucleus (**Figure 2.3H**). In contrast, Src$_{16}$GFP (net +5 positive charge) was found primarily at the plasma membrane and on a juxtanuclear compartment analogous to those seen with dually fatty acylated Yes- and FynGFP chimeras (**Figure 2.3K**). In those cases, it appears that the number of positively charged residues is a critical determinant for intracellular localization, and interestingly, the localization of Src_{16}GFP was indistinguishable from myristoylated and palmitoylated GFP chimeras.

To assess potential targeting differences between N-terminal combinations of myristate and palmitate and that of two palmitates (without myristate), the dipalmitoylated GAP-43GFP chimera was engineered and expressed in COS-7 cells. GAP-43GFP was localized to the plasma membrane and showed weak perinuclear punctate fluorescence in living cells (**Figure 2.3M**). This is in contrast to myristoylated and palmitoylated GFP constructs, which demonstrated a consistent focal perinuclear accumulation. In addition, the apparent inability of GAP-43(C3,4S)GFP to localize to membranes argues against an independent membrane-associating role for the putative second signal polybasic domain in this construct. All fatty acylation deficient chimeric GFPs and GFP alone were found throughout the cytosol and nucleus, with absence of fluorescence in vesicular lumenal areas (apparent negative staining) (**Figure 2.3, C, F, I, and L**).

2.4.5 Colocalization of Chimeric GFPs with Endosomes

In COS-7 cells, ERGIC, Golgi, TGN compartments and some endosomes are tightly packed in the perinuclear area. To improve visualization of colocalization data, NZ was used to disrupt microtubules and cause dispersion of these organelles. This allowed for better refinement of the colocalization of acylated GFP chimeras with various organelle markers. In order to identify the intracellular membranes targeted by the various chimeric GFPs, immunocytochemistry was utilized to colocalize GFP chimeras with known organelle markers. To find out whether YesGFP was located on endosomes, COS-7 cells expressing YesGFP were incubated with fluorescent DiI-LDL followed by NZ treatment. Similar subcellular localization of DiI-LDL internalized via the endocytic pathway and YesGFP are shown (**Figure 2.4, A and B**). The merged image (**Figure 2.4C**) showed significant colocalization of signals (yellow) in vesicular structures containing similar levels of fluorescence. Yes(C3S)GFP fluorescence was more dispersed and localized to various intracellular membranes, and was enriched in perinuclear vesicles (**Figure 2.4E**), whose distribution closely resembled that of internalized DiI-LDL (**Figure 2.4D**). When merged with the DiI-LDL signal, the resultant image shows bright perinuclear yellow vesicles, indicative of significant overlap of the vesicles with the endosomal marker (**Figure 2.4F**). Yes(G2A)GFP, a nonacylated chimera, was localized throughout the cell and did not colocalize with the endosomal marker (**Figure 2.5A-C**).

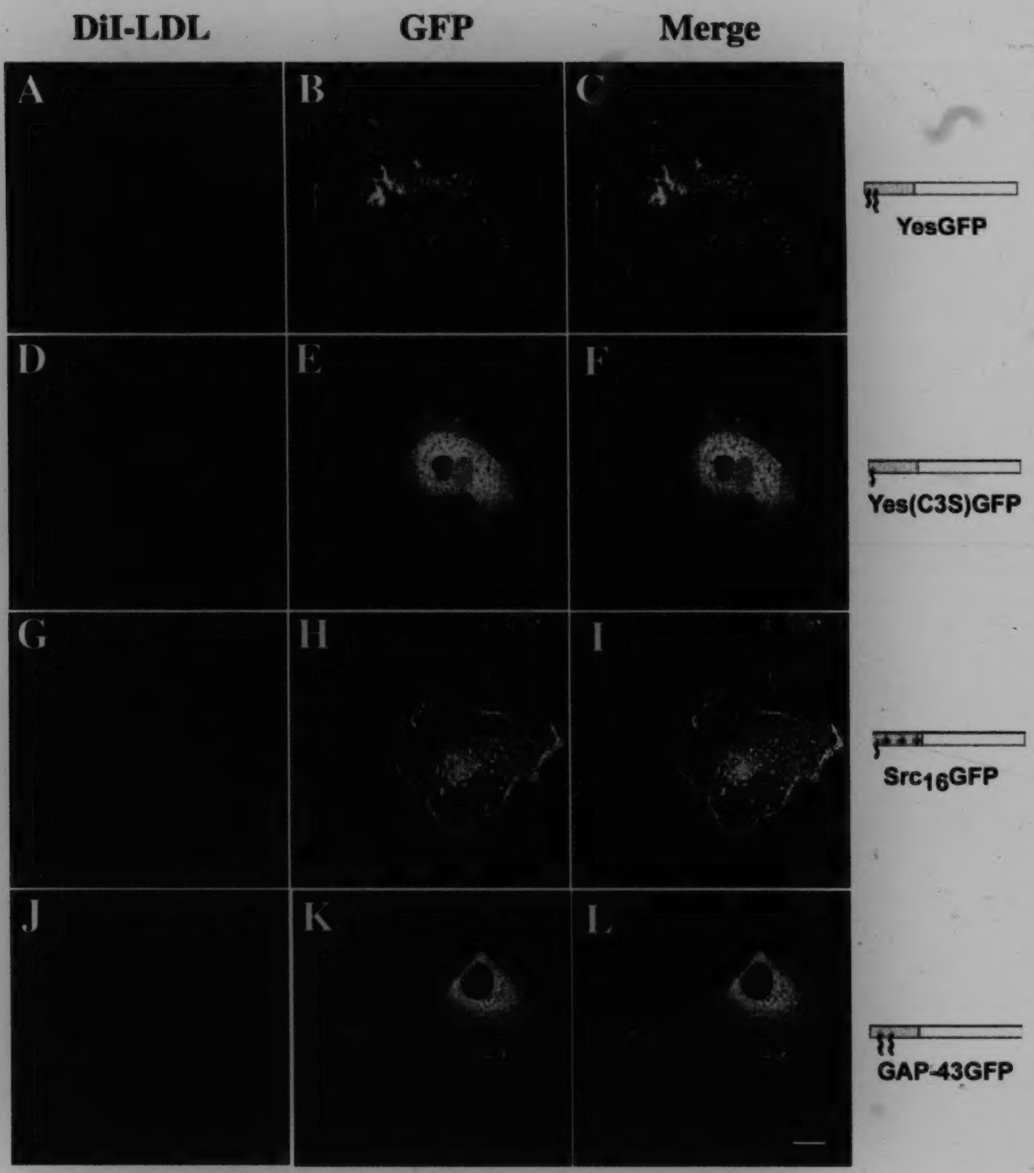
DiI-LDL
GFP
Merge
A
B
C
YesGFP
D
E
F
Yes(C3S)GFP
G
H
I
Src16GFP
J
K
L
GAP-43GFP

Figure 2.4. Colocalization of chimeric GFPs with endosomes in fixed COS-7 cells. Prior to immunocytochemistry, cells were treated for 1 h with 20 μM NZ. The GFP chimera fluorescence was detected by rabbit anti-GFP Ab followed by FITC-conjugated anti-rabbit Ab (A, D, G, and J). The same cells were incubated with DiI-LDL (B, E, H, and K). Merged images are shown on the right (C, F, I, and L). Bar, 10 μm. Model description as in **Fig. 2.3**; blue box, acylation sequence; green box, GFP.

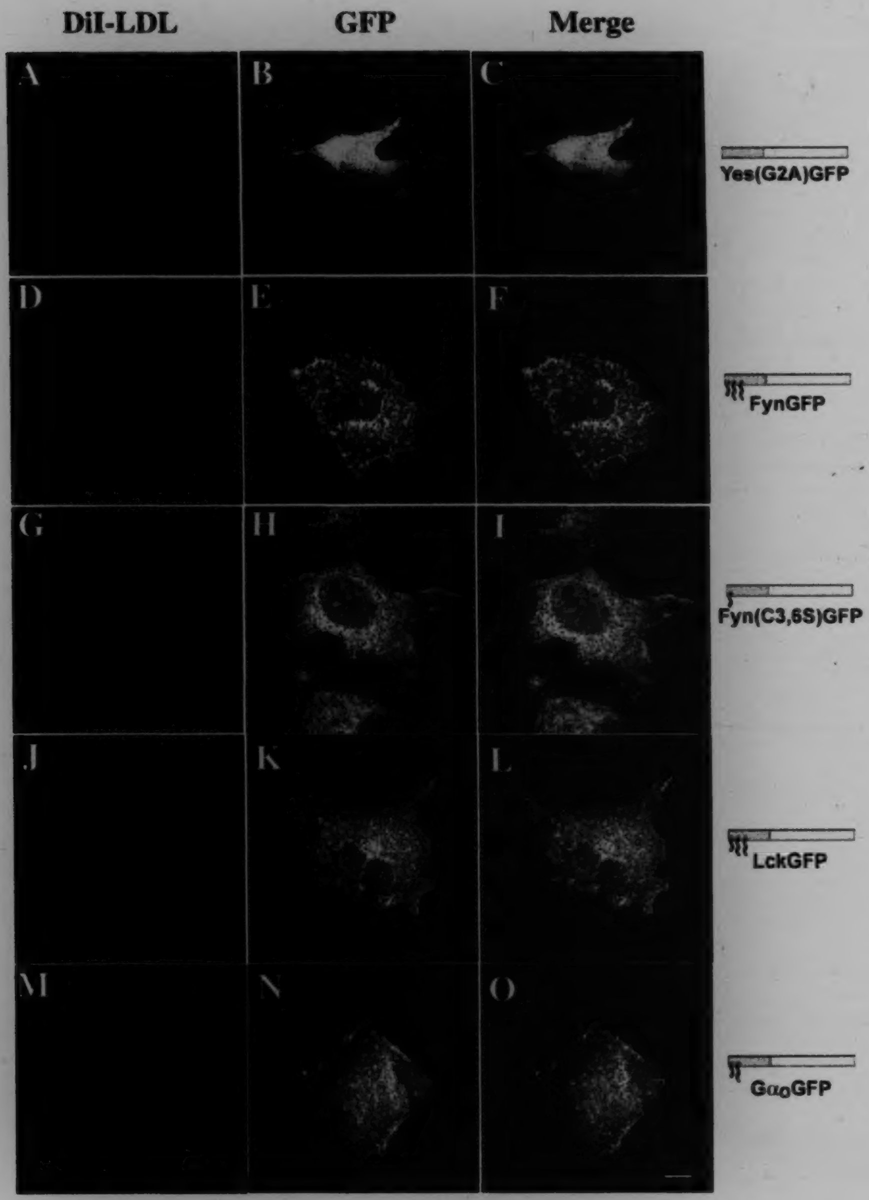
DiI-LDL
GFP
Merge
A
B
C
Yes(G2A)GFP
D
E
F
FynGFP
G
H
I
Fyn(C3,6S)GFP
J
K
L
LckGFP
M
N
O
Gα$_O$GFP

Figure 2.5. **Colocalization of chimeric GFPs with endosomes in fixed COS-7 cells.** Prior to immunocytochemistry, cells were treated for 1 h with 20 μM NZ. The GFP chimera fluorescence was detected by rabbit anti-GFP Ab followed by FITC-conjugated anti-rabbit Ab (A, D, G, J, and M). The same cells were incubated with DiI-LDL (B, E, H, K, and N). Merged images are shown on the right (C, F, I, L, and O). Bar, 10 μm. Model description as in **Fig. 2.3**; blue box, acylation sequence; green box, GFP.

The colocalization patterns of FynGFP and Fyn(C3,6S)GFP chimeras with the endosomal marker were indistinguishable from those obtained with the YesGFP chimeras (**Figure 2.5D-I**). Likewise, LckGFP and Gα_0GFP were also found at the plasma membrane and on endosomes (**Figure 2.5J-O**). The partial colocalization between the chimeric GFPs and the endosomal marker could be due to multiple types of endosomes that do not all contain endocytosed DiI-LDL, or simply differences in signal intensities of the two constituents in different vesicles.

Src$_{16}$GFP, which is myristoylated and contains a polybasic domain, also colocalized with the endosomal marker in a manner that was also apparently indistinguishable from the Yes- or FynGFPs (**Figure 2.4, G, H, and I**). In contrast with Yes-, Fyn-, and Src$_{16}$GFPs, dually palmitoylated (but not myristoylated) GAP-43GFP did not colocalize with the endosomal marker (**Figure 2.4, J, K, and L**).

When expressed at high levels, myristoylated plus palmitoylated or polybasic domain chimeric GFPs (*e.g.*, YesGFP and Src$_{16}$GFP, respectively), but not dipalmitoylated GAP-43GFP, showed partial colocalization with the integral membrane lysosomal marker CD63 in the perinuclear region (**Figure 2.6**). Hence, overexpression may allow some acylated/partially acylated chimeras to associate with lysosomes.

2.4.6 Colocalization of Chimeric GFPs with the Golgi and ER compartments

Intrinsic fluorescence of the YesGFP chimera in living cells is shown in **Figure 2.7A** and the Golgi apparatus staining is shown by the accumulation of BODIPY TR-ceramide (**Figure 2.7B**). The merged image (**Figure 2.7C**) demonstrates colocalization of the apparent Golgi signal with the intracellular fluorescence of YesGFP. In **Figure 2.7D**, Yes(C3S)GFP expression demonstrates targeting to various intracellular membranes. When merged with the Golgi marker signal (**Figure 2.7E**), the resultant image (**Figure 2.7F**) shows significant colocalization (yellow) with part of the Yes(C3S)GFP fluorescence. Nonacylated Yes(G2A)GFP is distributed throughout the cell (**Figure 2.7G**), and did not colocalize with BODIPY TR-ceramide (**Figure 2.7, H and I**). Similar results were found with dually acylated FynGFP and Lck GFP and myristoylated Fyn(C3,6S)GFPs (**Figure 2.8**).

Because of the transport requirement for internalization of BODIPY TR-ceramide and its possible localization to organelles other than the Golgi apparatus, Golgi colocalization was also assessed in fixed cells with an established resident Golgi marker, giantin (Linstedt and Hauri, 1993). In contrast to the results obtained with BODIPY TR-

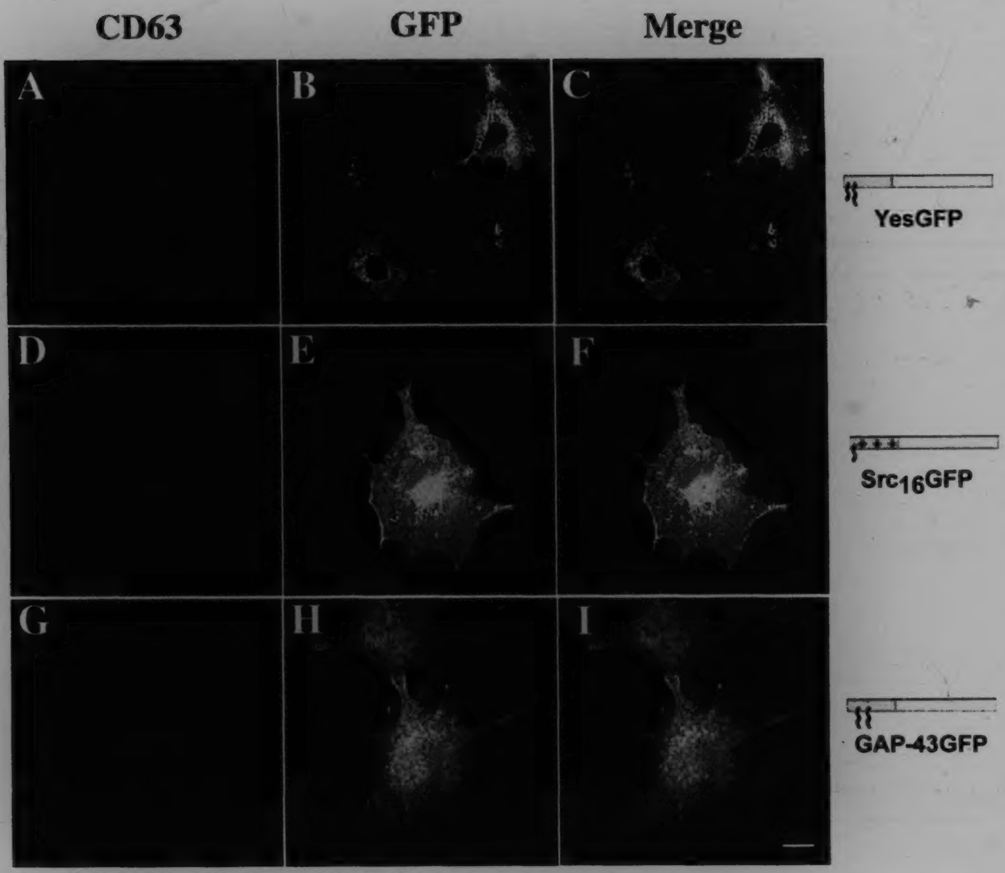
CD63
GFP
Merge
A
B
C
D
E
F
G
H
I
YesGFP
Src16GFP
GAP-43GFP

Figure 2.6. Colocalization of chimeric GFPs with lysosomes in fixed COS-7 cells. The lysosomal protein CD63 was detected by incubation with mouse anti-CD63 Ab followed by TR-conjugated anti-mouse Ab (A, D, and G). The same cells were incubated with rabbit anti-GFP Ab followed by FITC-conjugated anti-rabbit Ab (B, E, and H). Merged images are shown on the right (C, F, and I). Bar, 10 μm.

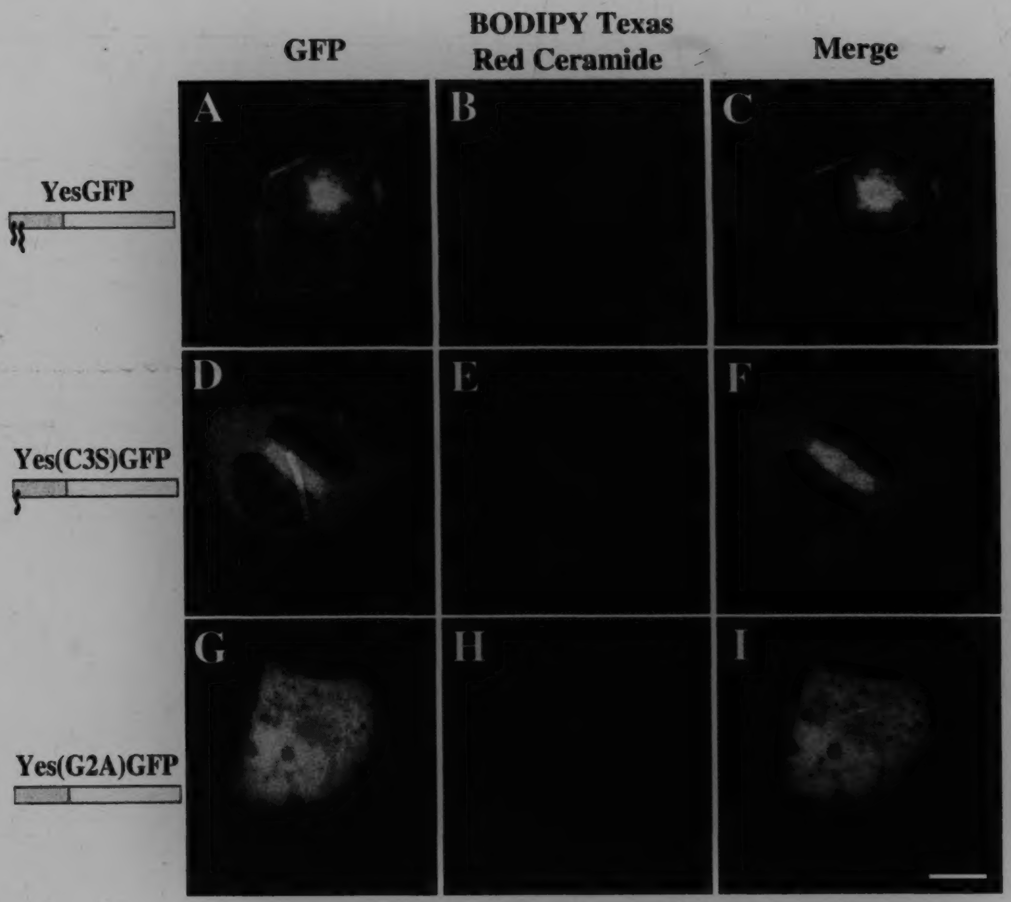
GFP
BODIPY Texas Red Ceramide
Merge
A
B
C
D
E
F
G
H
I
YesGFP
Yes(C3S)GFP
Yes(G2A)GFP

Figure 2.7. Colocalization of various chimeric GFPs with the Golgi marker BODIPY TR-ceramide in living COS-7 cells. GFP intrinsic fluorescence of YesGFP, Yes(C3S)GFP, and Yes(G2A)GFP is shown in (A), (D), and (G), respectively. The distribution of BODIPY TR-ceramide is shown in (B), (E) and (H). The merged images are shown in (C), (F), and (I). Bar, 10 μm.

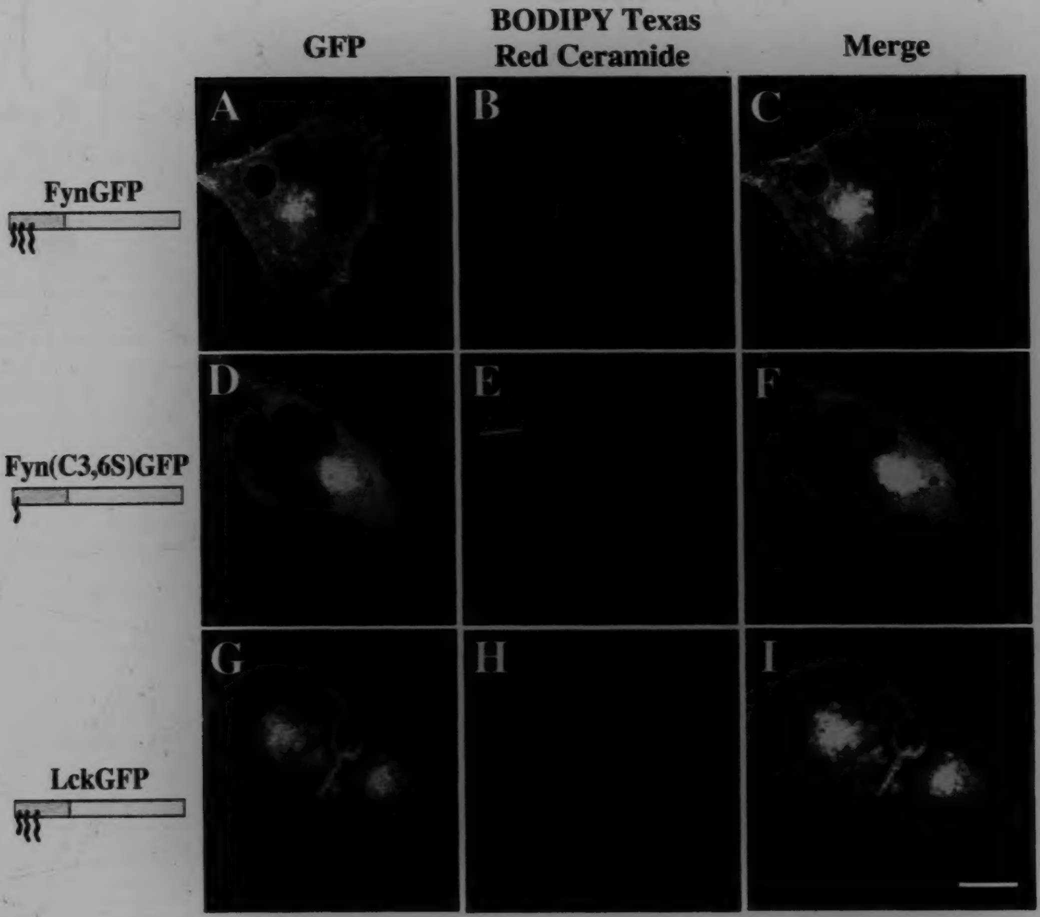
GFP
BODIPY Texas Red Ceramide
Merge
A
B
C
FynGFP
D
E
F
Fyn(C3,6S)GFP
G
H
I
LckGFP

Figure 2.8. Colocalization of various chimeric GFPs with the Golgi marker BODIPY TR-ceramide in living COS-7 cells. GFP intrinsic fluorescence of FynGFP, Fyn(C3,6S)GFP, and LckGFP is shown in (A), (D), and (G), respectively. The distribution of BODIPY TR-ceramide is shown in (B), (E) and (H). The merged images are shown in (C), (F), and (I). Bar, 10 μm.

ceramide in living cells, all constructs bearing myristate (*e.g.* Yes(C3S)GFP), myristate and palmitate (*e.g.* YesGFP) or myristate plus polybasic domain (*e.g.* Src_{16}GFP) did not colocalize with giantin. Typical results obtained with cells expressing YesGFP are shown in **Figure 2.9**. The giantin distribution is shown in red in **Figure 2.9 (A and D)** in the absence or presence of the microtubule disrupting agent NZ, respectively. In those cells, YesGFP signal depicted in green in **Figure 2.9 (B and E)**, when combined with the giantin signal, did not show any colocalization (**Figure 2.9, C and F**). In contrast, dipalmitoylated GAP-43GFP, which displayed only slight perinuclear fluorescence in living COS-7 cells, did show a significant perinuclear focal fluorescence in fixed and permeabilized cells (**Figure 2.9G**). When merged with the giantin signal (**Figure 2.9H**), there was significant overlap (yellow) in distribution with giantin (**Figure 2.9I**), although the GAP-43GFP signal did extend beyond that defined by giantin. GAP-43GFP localization was consistent with that of the Golgi but not restricted to this organelle. Paradoxically, upon NZ treatment, the perinuclear colocalization of GAP-43GFP (**Figure 2.9J**) with the giantin signal (**Figure 2.9K**) was abrogated (**Figure 2.9L**).

As the BODIPY-TR ceramide is internalized via endocytosis, in addition to the Golgi compartment, this ceramide analog might also be found in endosomes and areas of the trans-Golgi network (TGN). Using immunocytochemistry, we found that all acylated GFPs co-localized significantly with the endosomal marker DiI-LDL except GAP-43, which co-localized with the Golgi marker. Our data in **Figure 2.7** show overlap between acylated GFPs (which are found on internal endosomal membranes on as shown in **Figures 2.4** and **2.5**) and the BODIPY-TR ceramide. These data thus support the possibility that BODIPY-TR ceramide may accumulate in endosomes as well as the Golgi apparatus in COS-7 cells.

In order to identify the intracellular membranes targeted by the mutant chimeric GFPs, immunocytochemistry was utilized to colocalize GFP chimeras with a known ER marker, calreticulin. Yes(C3S)GFP was localized to various intracellular membranes (green, **Figure 2.10B**). When merged with the calreticulin staining (red, **Figure 2.10A**), the resultant image shows significant colocalization with the ER marker (yellow, **Figure 2.10C**). Identical results were seen with the Fyn(C3,6S)GFP chimera (**Figure 2.10G-I**). For YesGFP, the staining of intracellular vesicles identified as endosomes (**Figure 2.10E**) does not overlap with that of calreticulin (**Figure 2.10D**) as seen in the merged image (**Figure 2.10F**). Other chimeras demonstrating endosomal localization also did not colocalize with calreticulin (**Figure 2.11**).

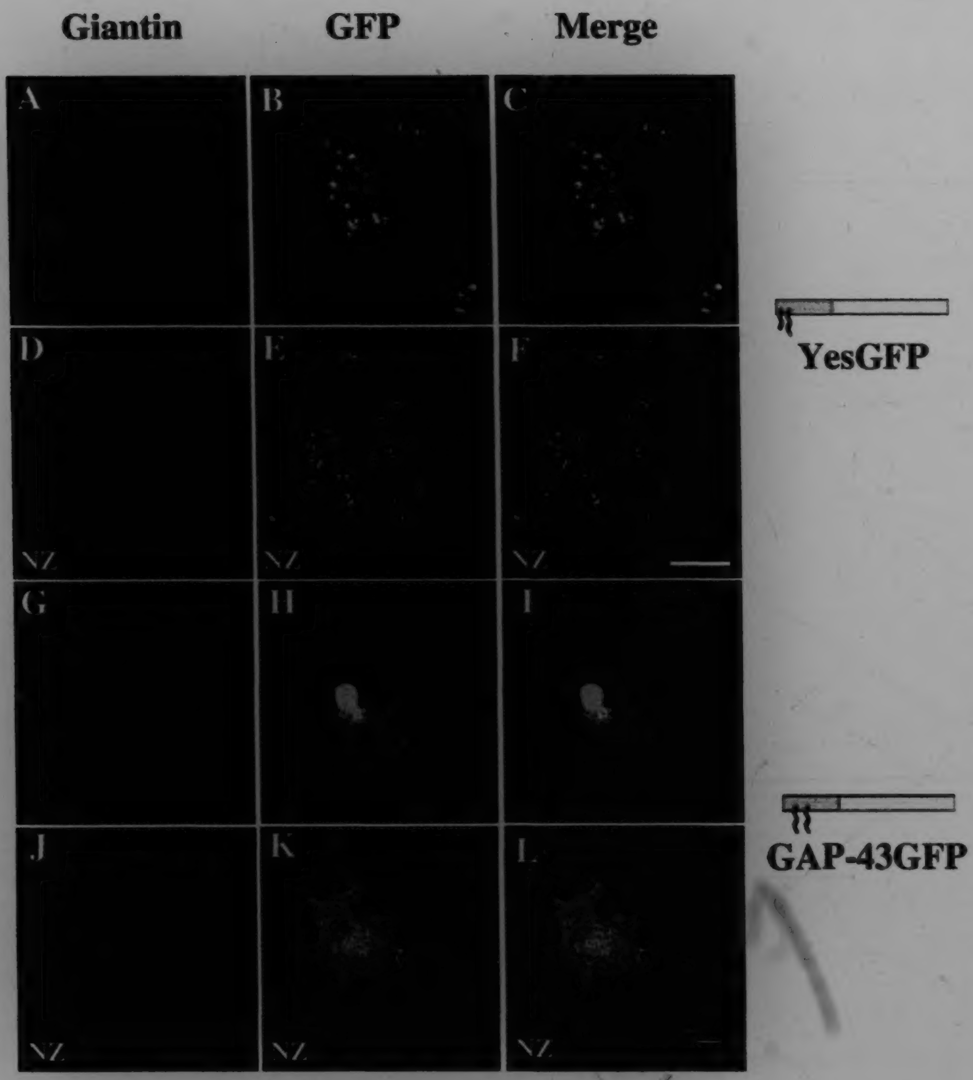
Giantin
GFP
Merge
A
B
C
D
E
F
NZ
NZ
NZ
G
H
I
J
K
L
NZ
NZ
NZ
YesGFP
GAP-43GFP

Figure 2.9. Colocalization of chimeric GFPs with the Golgi apparatus in fixed COS-7 cells. The GFP chimeras were detected by incubation with mouse anti-GFP Ab followed by FITC-conjugated anti-mouse Ab (A, D, G, and J). The same cells were incubated with rabbit anti-giantin Ab followed by TR-conjugated anti-rabbit Ab (B, E, H, and K). Merged images are shown on the right (C, F, I, and L). Images (A-C) and (G-I) represent untreated cells, and (D-F) and (J-L) represent cells treated for 1 h with 20 μM NZ before viewing. Bars, 10 μm.

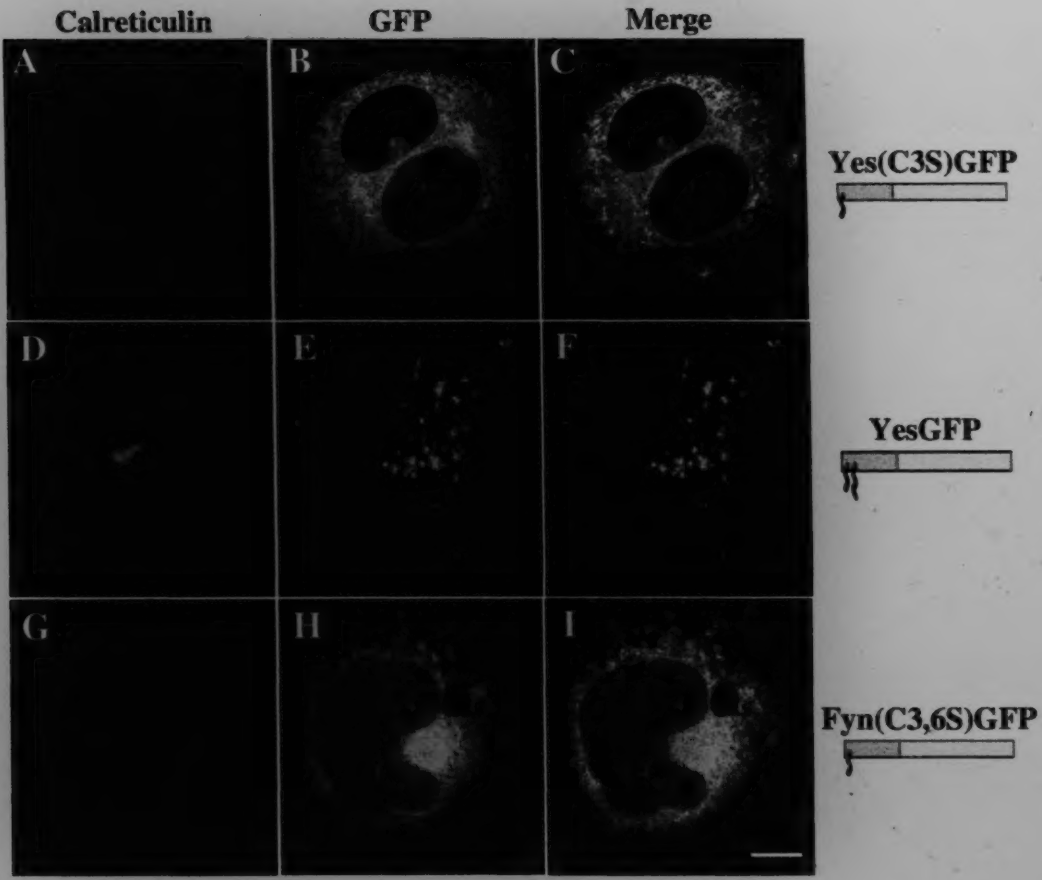
Calreticulin
GFP
Merge
A
B
C
D
E
F
G
H
I
Yes(C3S)GFP
YesGFP
Fyn(C3,6S)GFP

Figure 2.10. Colocalization of chimeric GFPs with the endoplasmic reticulum marker calreticulin in fixed COS-7 cells. The distribution of calreticulin, a lumenal protein of the ER, is detected by goat anti-calreticulin Ab followed by TR-conjugated donkey anti-goat Ab (A, D, and G). Yes(C3S)-, Fyn(C3,6S)- and YesGFP chimeras were detected by mouse anti-GFP Ab followed by FITC-conjugated anti-mouse Ab for YesGFP, Yes(C3S)GFP, and Yes(G2A)GFP are shown (B, E, and H). The merged images are shown (C, F, and I). Bar, 10 μm.

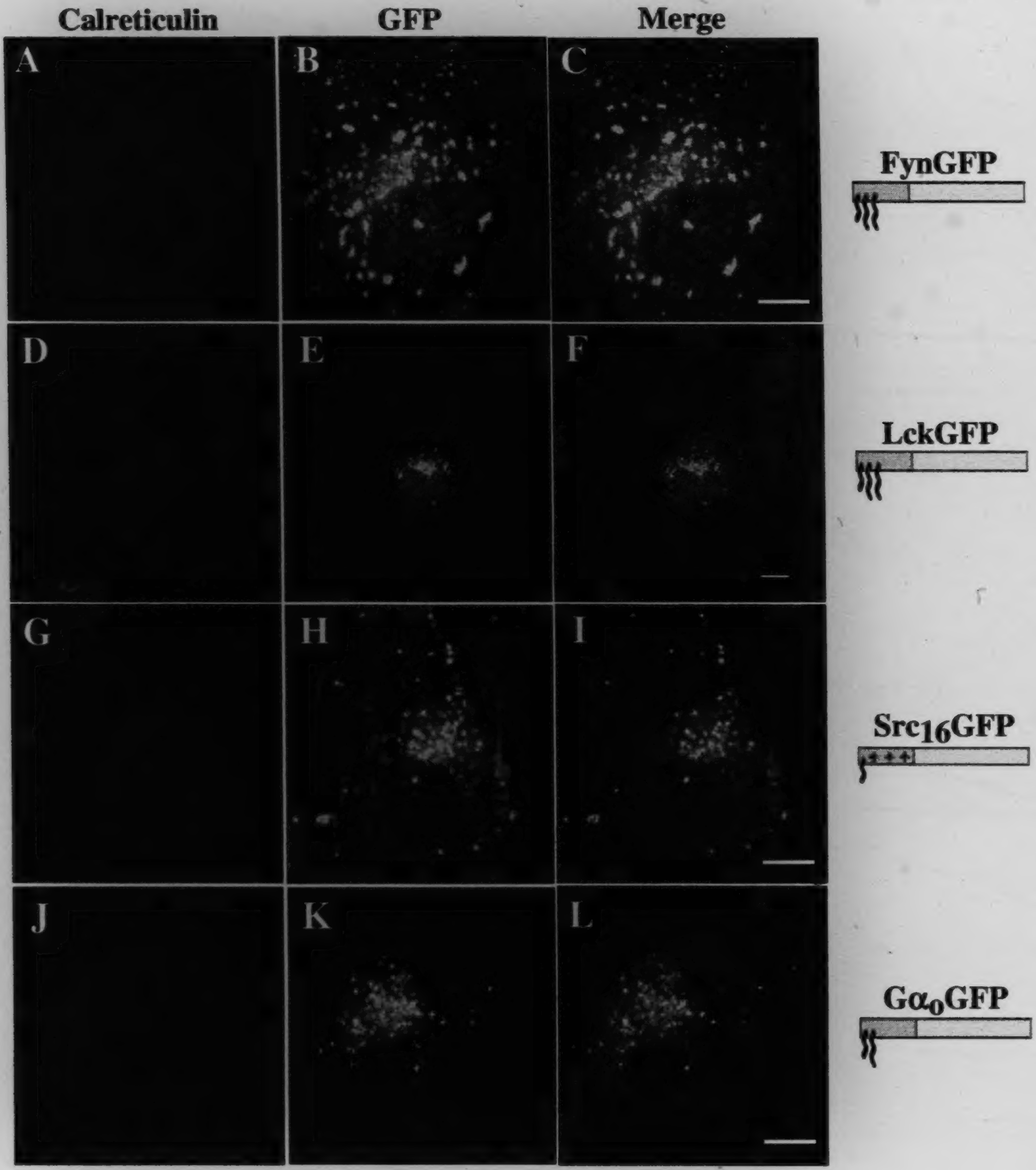
Calreticulin
GFP
Merge
A
B
C
D
E
F
G
H
I
J
K
L
FynGFP
LckGFP
Src16GFP
Gα0GFP

Figure 2.11. Colocalization of chimeric GFPs with the ER marker calreticulin in fixed COS-7 cells. The distribution of calreticulin is detected by goat anti-calreticulin Ab followed by TR-conjugated donkey anti-goat Ab (A, D, G, and J, respectively). Fyn-, Lck-, Src_{16}-, and $G\alpha_o$GFP chimeras were detected by mouse anti-GFP Ab followed by FITC-conjugated anti-mouse Ab. Merged images are shown (C, F, I, and L). Bars, 10 μm.

2.4.7 Colocalization of YesGFP and Yes protein tyrosine kinase

To assess whether the localization mediated by some of the fatty acylated domains appended to GFP reflected the localization of endogenous full-length proteins from which these domains were derived, double immunofluorescence study was carried out between YesGFP and the full-length Yes PTK, which is expressed in COS-7 cells. As seen in **Figure 2.12**, the signal provided by endogenous Yes (red, **Figure 2.12A**), and YesGFP (green, **Figure 2.12B**) were distributed in a similar fashion and, when merged, showed significant colocalization at the plasma membrane and in the perinuclear area (yellow, **Figure 2.12C**). Intracellular vesicular structures prominent in COS-7 cells expressing YesGFP chimera were absent or reduced in intensity in images of endogenous Yes PTK fluorescence.

2.5 DISCUSSION

2.5.1 N-terminal Fatty Acylated Sequences Mediate Differential Subcellular Localization

We dissected the molecular components of several N-terminally fatty acylated sequences required for plasma membrane localization and identified a series of intracellular membranes/vesicles where acylated GFPs were also found. These localization patterns could be categorized into two subsets. First, a myristoylated and palmitoylated GlyCys motif or a combination of myristate and significant polybasic domain (as in Src_{16}GFP) lead to endosomal and plasma membrane localization. A reduction in the length and net charge of the polybasic domain in SrcGFP lead to a more widespread localization to a variety of intracellular membranes including the nuclear envelope (*e.g.* Src_{14}GFP). Second, a doubly palmitoylated (but not myristoylated) motif (GAP-43GFP) conferred localization to the plasma membrane and the Golgi area, but not to endosomes.

In our system, the myristoylated and palmitoylated GlyCys motif (found in Fyn, Yes, Lck, and $G\alpha_o$) may be necessary and sufficient to confer localization to the plasma membrane and endosomes. Neither the number/position of palmitates nor the amino acids surrounding the acylation sites influenced localization in our system. Due to the fact that these various acylated sequences conferred similar localization properties to GFP, our results argue in favor of common biophysical properties of these acylated sequences specifying membrane localization. In agreement with this interpretation are recent reports demonstrating that the dually acylated N-terminal motif of $G\alpha_{i1}$ was sufficient to target GFP to caveolin-enriched DRMs (Galbiati *et al.*, 1999; Melkonian *et al.*, 1999). Also, potential differences in palmitate turnover on the various acylated GFP chimeras did not

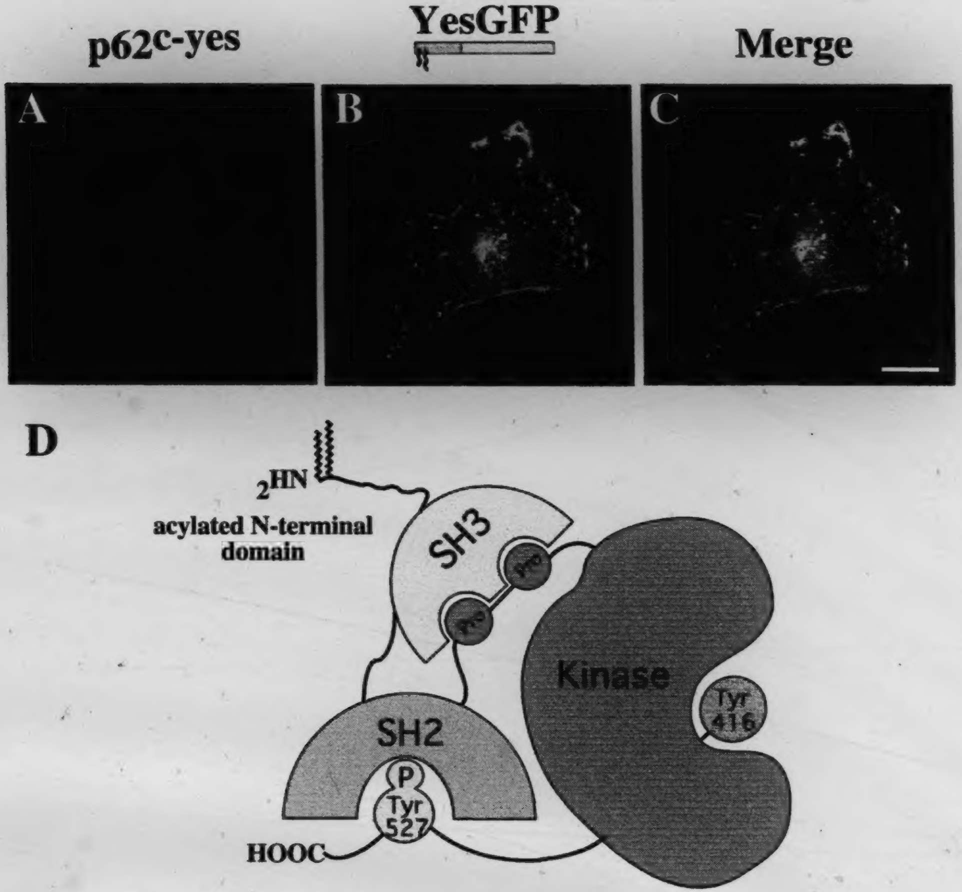
p62c-yes
YesGFP
Merge
A
B
C
D
2HN
acylated N-terminal domain
SH3
Pro
Pro
Kinase
Tyr 416
SH2
P
Tyr 527
HOOC

Figure 2.12. NH_2-terminal domain YesGFP chimera is localized similarly to endogenous full-length Yes PTK. (A), endogenous Yes PTK was detected with mouse anti-Yes Ab followed by TR-conjugated anti-mouse Ab. (B), YesGFP chimera detected with rabbit anti-GFP Ab followed by FITC-conjugated anti-rabbit Ab. (C), merged image showing the significant colocalization between the two proteins. (D), model of the 3-D crystal structure of the Src PTK family showing the intramolecular protein domain interactions and putative exposure of the acylated N-terminal region. Bar, 10 μm.

alter either apparent subcellular localization as judged by confocal microscopy, or the ability of chimeric GFPs to associate productively with biological membranes.

Our data clearly demonstrate that the first 11 amino acids of the Yes PTK are sufficient to localize GFP in membrane domains where endogenous full-length Yes PTK can be found. As well, the localization of our dually acylated chimeras in COS-7 cells agree with several previously described studies on the subcellular localization of corresponding full length signaling proteins. Src family PTKs and G protein α subunits are targeted to the plasma membrane and intracellular membranes including punctate structures, TGN, endosomes and Golgi area in many cell types (Krueger *et al.*, 1991; Ley *et al.*, 1994; Denker *et al.*, 1996; van't Hof and Resh, 1997; Wolven *et al.*, 1997; Galbiati *et al.*, 1999). Also, N-terminal Lck and Fyn fusion chimeras are targeted in a similar fashion (Wolven *et al.*, 1997; Zlatkine *et al.*, 1997). However, in contrast to these findings, endogenous Fyn targets exclusively to the centrosomal area in hematopoietic cells (Ley *et al.*, 1994; Campbell *et al.*, 1998), and Lck is found only at the plasma membrane in NIH-3T3 cells. CD4 cotransfection (a plasma membrane receptor which binds Lck) can abrogate TGN/endosomal-targeted Lck in HeLa cells (Bijlmakers *et al.*, 1997).

Taken together, we show that N-terminal acylation is a key spatial determinant resulting in proper plasma membrane and endosome localization but is likely not the sole mediator of subcellular localization of the corresponding full-length acylated signaling proteins within cells. As such, cell type specific factors, protein-protein interaction modules downstream of the acylation sites, the status of palmitoylation, or even protein tertiary conformation may play essential roles in determining the net effect on protein localization. To illustrate this, when Src family PTKs are not activated, the binding sites of SH3 and SH2 domains are occupied by an intramolecular proline rich region and C-terminal regulatory phosphotyrosine residue, respectively, in the Hck and Src PTK crystal structures (see model, **Figure 2.12D**) (Sicheri and Kuriyan, 1997; Sicheri *et al.*, 1997; Xu *et al.*, 1997). As such, the SH3 and SH2 domains cannot account for subcellular localization of Src-related PTKs in the inactive state. This strengthens the importance of fatty acylation in proper localization. Furthermore, controlling the presence of palmitates on dually acylated signaling PTKs, or phosphorylation state of serine residues within the polybasic region of the c-Src N-terminus, could potentially allow shuttling between membranes of various organelles and the plasma membrane.

Myristoylated $Src_{16}GFP$ containing additional positively charged residues was capable of specific plasma membrane and endosome localization with no apparent ER localization, in comparison to $Src_{14}GFP$. Interestingly, full-length c-Src has been located at the plasma membrane and endosomes (Kaplan *et al.*, 1992; David-Pfeuty *et al.*, 1993;

Luttrell *et al.*, 1999). Changes in the states of activation and/or phosphorylation of c-Src may result in regulated differential localization (Kaplan *et al.*, 1992; Silverman and Resh, 1992; Schwartzberg, 1998). Clearly, though, the N-terminus alone plays an important role in PTK localization.

The similarity in localization mediated by a combination of myristoylation and palmitoylation and myristoylation plus polybasic domain on GFP was intriguing. It suggests that the inner leaflet of the plasma membrane and the outer leaflet of the endosome exhibit a combination of hydrophobic and electrostatic properties that can accommodate both types of acylated proteins. The presence of negatively charged phospholipids (*e.g.* phosphatidylserine) being concentrated in the inner leaflet of the plasma membrane is well-documented (Devaux, 1991; Zwaal and Schroit, 1997). Because a significant portion of these negatively charged phospholipids are known to contain two saturated acyl chains (Holub, 1980), this population could preferentially be found in glycosphingolipid-enriched lipid raft or DRM domains (Harder and Simons, 1997). Through the formation of a liquid-ordered membrane phase, these domains are believed to selectively recruit lipids with saturated acyl chains and proteins modified by such lipids (Brown and London, 1998b). In addition, endosomal membranes, which can support a liquid-ordered membrane phase (Brown and London, 1998a), are known to be the most net negatively charged of all cellular membranes, as witnessed by their characteristic furthest migration towards the anode in free flow electrophoresis experiments (Cavenagh *et al.*, 1996). These points illustrate that acylation coupled with a polybasic region could lead to targeting to lipid raft domains in the plasma membrane or endosomes analogous to myristoylated and palmitoylated proteins. Although not described as a DRM associating signal (Melkonian *et al.*, 1999), c-Src containing myristate and a polybasic domain has been shown to cofractionate and colocalize with caveolin-1 (Li *et al.*, 1996; Song *et al.*, 1997), which is often enriched in DRMs.

The dipalmitoylation motif, present in GAP-43-GFP, appears to be equally effective in directing proteins to the plasma membrane. Immunofluorescence results using a polyclonal GFP Ab (which would detect both mature fluorescent GFP molecules as well as a nascently synthesized nonfluorescent population) demonstrate that there is a significant intracellular fluorescence concentration, which colocalizes with the Golgi marker, giantin. Golgi targeting by wild type and N-terminal chimeras of GAP-43 has been documented by others in COS-7 cells (Liu *et al.*, 1993, 1994; Arni *et al.*, 1998). The association of related N-terminally palmitoylated growth cone proteins, SCG-10 and SNAP-25, with the Golgi compartment has also been reported (Di Paolo *et al.*, 1997; Gonzalo and Linder, 1998). Upon NZ treatment, Golgi localization of GAP-43GFP was abrogated but plasma membrane localization was not. This indicates that GAP-43 may be palmitoylated at the plasma membrane and that an intact microtubule network may be required for accumulation of the chimera on the Golgi membranes. Potentially, NZ treatment may also impede palmitoylation of newly synthesized GAP-43GFP. As such, our localization results with the GAP-43GFP chimera are consistent with those reported by others and are consistent with a role for dual palmitoylation in Golgi area localization.

2.5.2 Myristoylation is sufficient for intracellular membrane association and nuclear exclusion

The inability of myristoylated chimeras to associate productively with the plasma membrane, which represents 5-10% of total membranes in cultured cells (Griffiths *et al.*, 1984; Alberts *et al.*, 1994), is not well understood. Weak membrane-binding properties of myristate may allow the rapid dissociation of myristoylated proteins from a variety of membranes (Shahinian and Silvius, 1995). As such, rapid dissociation may represent a means to sample different membranes. Thus, soon after cotranslational myristoylation, more abundant intracellular membrane fractions could sequester most of the myristoylated chimera away from the plasma membrane. To illustrate this possibility, we saw very significant localization of various myristoylated GFPs on ER membranes, which represent up to 60% of total cellular membranes (Alberts *et al.*, 1994).

Due to the detection capability of the confocal microscope and the significant chimeric protein production in our system, we believe that the presence of GFP chimeras at the plasma membrane should have been detected if myristoylation provided a random means of membrane sampling. Potentially, rapid endocytic events may remove myristoylated GFPs from the inner leaflet of the plasma membrane. This possibility is confirmed by our results: myristoylated GFPs colocalize with the endocytic marker DiI-LDL. Due to the extensive and dense perinuclear accumulation of a number of myristoylated GFP constructs, some of the noted apparent colocalization with organelle markers may be due to the juxtaposition of the two signals. In addition, our myristoylated GFPs did not colocalize with the Golgi marker giantin. Thus, in addition to random sampling of membranes, our data suggest that an exposed myristate (as found in our GFP chimeras) could confer specific localization information that allows enrichment in ER and endosomal membranes. This is in contrast to cytosolic myristoylated proteins in which the myristate is sequestered in a hydrophobic pocket (*e.g.* calcineurin and cAMP-dependent protein kinase), and unable to influence membrane association (Zheng *et al.*, 1993; Griffith *et al.*, 1995).

Our observations are thus consistent with myristoylation acting as an intracellular membrane-associating signal. While the membrane association of a myristoylated fluorescent peptide in the absence of palmitoylation or polybasic residues has been demonstrated to be transient (Shahinian and Silvius, 1995), myristoylated GFPs in our study are found associated primarily with membranes as judged by confocal microscopy of living cells and subcellular fractionation. As such, our data are not necessarily consistent with previous data obtained *in vitro*. Inside the living cell, myristate might contribute a better association with membranes than *in vitro*, possibly via association with DRMs or lipid rafts. Consistent with this possibility, an N-myristoylated tumor necrosis factor fusion protein was completely bound to dipalmitoylphosphatidylcholine (DPPC) liposomes whereas the G2A mutant chimera did not (Utsumi *et al.*, 1996). These DPPC liposomes would promote a liquid-ordered membrane phase reminiscent of DRMs or rafts (Brown and London, 1998a).

Intracellular membrane association may explain the nuclear exclusion of acylated proteins, as, other than the nuclear envelope, the nucleus is devoid of membranes. Sequestration of multiple substrate signaling proteins (like c-Src) outside the nucleus may be an important factor in preventing interference with nuclear signaling, as proposed previously (David-Pfeuty *et al.*, 1993).

2.5.3 Evidence for Multiple Palmitoylation Mechanisms

The plasma membrane/endosomal localization observed with myristoylated/palmitoylated chimeras versus plasma membrane/Golgi region localization observed with dually palmitoylated chimeras suggest that two PAT activities may exist. In the cases where covalent addition of palmitate would act as the retention signal, the localization of putative PAT(s) could play an active role in plasma membrane, endosome, and Golgi localization. Consistent with this possibility, a PAT activity that palmitoylates G protein α subunits is concentrated in the plasma membrane/endosome fraction (Dunphy *et al.*, 1996). In addition, we have also characterized a PAT activity that palmitoylates myristoylated PTKs that was enriched in both crude plasma membrane and Golgi membrane fractions and absent in the ER fraction isolated from rat liver (Berthiaume and Resh, 1995). A second PAT activity presumed to be in the ERGIC/*cis*-Golgi has been shown to palmitoylate viral glycoproteins and presumably endogenous cellular proteins such as GAP-43, SCG-10, or SNAP-25 (Veit *et al.*, 1996b; McLaughlin and Denny, 1999). These proteins are known to use the secretory pathway to get to the plasma membrane and are known to associate with the Golgi/TGN (Di Paolo *et al.*, 1997; Gonzalo and Linder, 1998). It is likely that our GAP-43GFP chimera may be acylated by this PAT activity.

2.5.4 Mechanisms of Fatty Acylation-Dependent Subcellular Localization

In terms of area and mass, the plasma membrane and endosomes represent only a minor membrane component in most eukaryotic cells. Thus, the ~20 to 50-fold enrichment of dual signal containing GFPs in the plasma membrane/endosomes in comparison to other intracellular membranes demonstrates the specificity generated by combining fatty acids and polybasic domains at the N-termini of proteins. Overall, our subcellular localization data are consistent with the kinetic bilayer trapping mechanism (Shahinian and Silvius, 1995), with palmitoylation acting as a plasma membrane retention signal, and with an enhanced function for myristoylation acting as a possible retention signal for endosomes or ER membranes. In the kinetic bilayer trapping model, singly acylated proteins or peptides are postulated to randomly and rapidly diffuse on and off various intracellular membranes until they are retained at their final destination via covalent addition of a second lipid (often palmitate) (Schroeder *et al.*, 1996). In addition, also consistent with the kinetic bilayer trapping mechanism, the polybasic domain in the Src chimera could act as a retention signal on negatively charged endosomes and inner plasma membrane leaflet.

CHAPTER 3

N-TERMINAL PROTEIN ACYLATION CONFERS LOCALIZATION TO CHOLESTEROL, SPHINGOLIPID-ENRICHED MEMBRANES BUT NOT TO LIPID RAFTS/CAVEOLAE

A version of this chapter has been published. McCabe, J.B., and Berthiaume, L.G. (2001). N-terminal protein acylation confers localization to cholesterol, sphingolipid-enriched membranes but not to lipid rafts/caveolae. Mol. Biol. Cell *12*, 3601-3617.

3.1 ABSTRACT

When variably fatty acylated N-terminal amino acid sequences were appended to a green fluorescent reporter protein (GFP), chimeric GFPs were localized to different membranes in a fatty-acylation-dependent manner. To explore the mechanism of localization, the properties of acceptor membranes and their interaction with acylated chimeric GFPs were analyzed in COS-7 cells. Myristoylated GFPs containing a palmitoylated or polybasic region co-localized with cholesterol and ganglioside GM_1, but not with caveolin, at the plasma membrane and endosomes. A dipalmitoylated GFP chimera colocalized with cholesterol and GM_1 at the plasma membrane and with caveolin in the Golgi region. Acylated GFP chimeras did not cofractionate with low-density caveolin-rich lipid rafts prepared using TX-100 or detergent-free methods. All GFP chimeras, but not full-length $p62^{c\text{-}yes}$ and caveolin, were readily solubilized from membranes using various detergents. These data suggest that while N-terminal acylation can bring GFP to cholesterol and sphingolipid-enriched membranes, protein-protein interactions are required to localize a given protein to detergent-resistant membranes (DRMs) or caveolin-rich membranes. In addition to restricting acceptor membrane localization, N-terminal fatty acylation could represent an efficient means to enrich the concentration of signaling proteins in the vicinity of DRMs and facilitate protein-protein interactions mediating transfer to a detergent-resistant lipid raft core.

3.2 INTRODUCTION

Numerous signaling proteins contain combinations of covalently attached fatty acids (myristate and palmitate) at their N-termini. Such proteins include several cellular Src-related protein tyrosine kinases (PTKs: Yes, Fyn, Lyn, Lck, Hck, Fgr, and Yrk), G protein α subunits ($G\alpha_i$, $G\alpha_o$, $G\alpha_z$, yeast GPA1), endothelial nitric oxide synthase (ecNOS), and A-kinase anchoring protein AKAP18 (Dunphy and Linder, 1998; Resh, 1999). In addition, several proteins are singly myristoylated and possess an adjacent or distant polybasic amino acid domain. These include the PTKs Src and Blk, MARCKS, and HIV-1 Nef and Gag (Resh, 1999). Other proteins have been shown to contain two or more covalently linked palmitates at their N-termini, as in GAP-43, $G\alpha_q$, PSD-95, and regulator of G protein signaling (RGS) isoforms (Dunphy and Linder, 1998; Resh, 1999). In all cases, a cooperative interaction of at least two signals (including multiple N-terminal acylation, or an acylation site juxtaposed to a polybasic domain) is required to promote efficient membrane association of the modified (Dunphy and Linder, 1998; Johnson and Cornell, 1999; McCabe and Berthiaume, 1999; Resh, 1999). This cooperative mechanism is also used by Ras superfamily proteins (including H-, K-, and N-Ras) at the C-terminus using different lipid anchors (farnesyl and geranylgeranyl) coupled with polybasic regions and/or palmitoylation (Choy *et al.*, 1999; Magee and Marshall, 1999).

Recently, various combinations of lipids attached to proteins have been shown to specify lipidation-dependent enrichment of lipidated proteins to certain membranes (Choy *et al.*, 1999; McCabe and Berthiaume, 1999). These recent advances provided a novel understanding of the existence of different lipid anchors attached to a variety of proteins. It appears that in the many cases studied to date, differential lipidation allows for localization to different acceptor membranes. Thus, lipidated sequences seem to act as novel types of signal or retention sequences. We demonstrated that GFP chimeras containing myristoylation and palmitoylation signals or a myristoylation signal juxtaposed to a polybasic region at the N-termini were localized similarly to the plasma membrane and endosomes in COS-7 cells. In contrast, a dipalmitoylated, nonmyristoylated GFP chimera was localized to the Golgi region and plasma membrane. Removal of the palmitoylation signal in myristoylated and palmitoylated proteins resulted in loss of localization specificity, and led to relocalization to a variety of membranes. The fact that all myristoylated and palmitoylated GFP chimeras localized similarly to endosomes and plasma membrane ruled out the involvement of surrounding amino acids in subcellular localization. Thus, the combinatorial identity of N-terminal membrane-associating signals was likely important for subcellular distribution of these proteins.

Evidence has accumulated over the past few years supporting the existence of discrete membrane microdomains known as lipid rafts, and also referred to as detergent-resistant membranes (DRMs), detergent-insoluble glycosphingolipid-rich fractions (DIGs), and caveolin-enriched membranes (Parton and Simons, 1995; Simons and Ikonen, 1997; Anderson, 1998; Brown and London, 1998b, a; Galbiati *et al.*, 1999a; Resh, 1999). These membrane subdomains have been reported to be enriched in glycosphingolipids, free cholesterol and saturated phospholipids (Harder and Simons, 1997; Fridriksson *et al.*, 1999), and in some cases several lipidated proteins including non-receptor protein tyrosine kinases and caveolin (Kurzchalia and Parton, 1999; Oh and Schnitzer, 1999). These membrane rafts have been described as liquid-ordered (l_o) phase lipid domains dispersed in a liquid crystalline (or liquid disordered, l_d) lipid bilayer (Brown and London, 1998b, a). Membrane raft size has been shown to vary from 70 to 370 nm in diameter (Varma and Mayor, 1998; Hooper, 1999; Jacobson and Dietrich, 1999). A collection of data suggests that rafts are small and dynamic, but can be stabilized into caveolae or larger domains by specific proteins as well as by antibody crosslinking or patching (Harder *et al.*, 1998; Rietveld and Simons, 1998). Lipid rafts from different origins have been shown to be similar in many properties, but unique in others (*e.g.* constituent proteins or lipids), and support the existence of many kinds of lipid rafts (Parkin *et al.*, 1996; Iwabuchi *et al.*, 1998; Kurzchalia and Parton, 1999; Waugh *et al.*, 1999).

Caveolin, a 21 kDa palmitoylated integral membrane cholesterol-binding protein (Murata *et al.*, 1995), has been utilized as a marker for caveolae (Song *et al.*, 1996; Song *et al.*, 1997; Orlandi and Fishman, 1998; Oh and Schnitzer, 1999). Due to the comparable properties (detergent insolubility, lipid constituents, and buoyancy) between caveolae and DRMs/lipid rafts, caveolin has also been utilized as a marker of DRMs/lipid rafts during isolation. However, selective isolation procedures indicated that caveolae do not equate with detergent-insoluble lipid rafts (Hooper, 1999), and that non-caveolar lipid rafts do exist as separate entities (Iwabuchi *et al.*, 1998). Caveolin possesses a complex intracellular cycling pathway involving plasma membrane, caveolae, ER, ERGIC, and Golgi, and cytosol, and as such is found in a variety of membranes (Conrad *et al.*, 1995; Uittenbogaard *et al.*, 1998).

N-terminally acylated proteins, such as Yes, GAP-43, $G\alpha_i$, $G\alpha_o$ and Fyn, have been shown to be enriched in 4°C TX-100 insoluble structures (Arreaza *et al.*, 1994; van't Hof and Resh, 1997; Arni *et al.*, 1998; Galbiati *et al.*, 1999a; Melkonian *et al.*, 1999). In those proteins, loss of acylation resulted in the loss of functional fractionation to lipid rafts. In most cases, two saturated acyl chains are believed to be required for partitioning of the proteins into liquid-ordered cholesterol and glycosphingolipid-enriched membrane microdomains (Schroeder *et al.*, 1994; Arni *et al.*, 1998; Schroeder *et al.*, 1998). Proteins modified by single acyl chains or bulky, branched hydrophobic lipids (farnesyl and geranylgeranyl) are not believed to partition favorably into liquid ordered membrane rafts (Melkonian *et al.*, 1999; Moffett *et al.*, 2000). Other reports show that prenyl groups and myristate alone are sufficient to enrich in lipid rafts as defined by cofractionation with caveolin enriched membranes (Song *et al.*, 1997; Michaely *et al.*, 1999).

Several studies have demonstrated that mutations abolishing protein acylation resulted in a loss of function and fractionation to lipid rafts (reviewed in (Dunphy and Linder, 1998; Resh, 1999). We are investigating whether the addition of acylated sequences to an otherwise cytosolic reporter protein would confer a gain of function and allow partitioning or fractionation into lipid rafts. Furthermore, to investigate the mechanism of lipidation-dependent localization of variably acylated GFPs, we characterized the biochemical composition of acceptor membranes. Myristoylated and palmitoylated or polybasic region-containing GFPs colocalized with cholesterol and sphingolipid-enriched membranes, but not with caveolin. A dipalmitoylated (but not myristoylated) GFP did colocalize with caveolin intracellularly. We also show that N-terminal acylation signals appended to GFP are not sufficient to enrich the resulting chimeric GFPs in lipid rafts prepared using detergent and detergent-free methods. When the solubilization of chimeric

GFPs was analyzed by a kinetic *in situ* extraction assay, acylated GFPs were found to be readily solubilized, as opposed to the detergent resistant fatty acylated endogenous proteins caveolin and PTK $p62^{c\text{-}yes}$. Our findings collectively support a model where N-terminal acylation coupled with protein-protein interactions or perhaps additional protein-lipid interactions are required to bring these detergent-soluble proteins from cholesterol-, sphingolipid-enriched membranes to DRMs.

3.3 MATERIALS AND METHODS

3.3.1 Plasmids

The pCMV5 mammalian expression vector (Andersson *et al.*, 1989) containing various acylated 11-16 amino acid sequences appended to the S65T mutant of GFP (YesGFP, Yes(C3S)GFP, Yes(G2A)GFP, $G\alpha_o$GFP, GAP-43GFP, Src_{16}GFP, FynGFP, and LckGFP) and GFP alone were from a previous study (McCabe and Berthiaume, 1999), and are depicted in **Table 3.1**. The acylation status of these constructs has been previously determined to conform to predicted modification based on metabolic labeling and subcellular fractionation studies in our previous report (McCabe and Berthiaume, 1999).

3.3.2 Cell Lines, Antibodies, and Reagents

COS-7 cells were from the American Type Culture Collection (USA), and were maintained in 10% fetal bovine serum in Dulbecco's Modified Eagle's Medium (Gibco BRL) with 100 U/ml penicillin G sodium, 100 μg/mL streptomycin sulfate plus 2 mM L-glutamine (Sigma), and passed twice per week using a 0.25% trypsin/1 mM EDTA wash (Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Rabbit polyclonal anti-GFP antibody (Ab) was developed in our laboratory using highly purified recombinant GFP made in *E. coli* as antigen; mouse monoclonal anti-GFP was from Chemicon. Mouse monoclonal anti-Yes PTK, anti-caveolin-1 (clone 2297), anti-syntaxin 6, anti-early endosome-associated protein-1 (EEA1) antibodies and rabbit polyclonal anti-caveolin antibody were from Transduction Labs. Mouse monoclonal antibody to the cation independent mannose 6-phosphate receptor (CI-MPR) was from Affinity Bioreagents. Texas Red conjugated transferrin (TR-Tf), and Prolong Antifade

Table 3.1: Colocalization of acylated proteins and lipid raft constituents.

Protein	Membrane Associating Signals[1]	Cellular Localization[2]	Colocalization[3] with caveolin	Colocalization[3] with cholesterol	Colocalization[3] with GM$_1$	Lipid Raft Association[4]
YesGFP	myr+palm (3,5)[5]	pm + endo	–	+	+	–
Yes(C3S)GFP	myr	endo + ER	–	+	+	–
Yes(G2A)GFP	none	cyto, nuc	–	–	–	–
FynGFP	myr+palm (3,6)	pm + endo	–	+	+	–
Gα_oGFP	myr+palm (3)	pm + endo	–	+	+	–
LckGFP	myr+palm (3,5)	pm + endo	–	+	+	–
Src$_{16}$GFP	myr+pos	pm + endo	–	+	+	–
GAP-43GFP	palm+pos (3,4)	pm + Golgi	+(Golgi)	+	+	–
GFP	none	cyto, nuc	–	–	–	–
Caveolin	PTM+palm	pm/Golgi/ER/cyto[8]	+	+[6]	+[6]	+
p62$^{c\text{-}yes}$	myr+palm	pm + endo[8]	+	+[6]	ND[7]	+

1 Known cooperative signals responsible for membrane association. myr: myristoylation; pal: palmitoylation; pos: polybasic region; PTM: partial transmembrane domain.

2 As determined in this and a previous study (McCabe and Berthiaume, 1999). pm: plasma membrane; endo: endosomes; ER: endoplasmic reticulum; cyto: cytosol; Golgi: Golgi region.

3 Immunofluorescence using anti-GFP and anti-caveolin Abs. Cholesterol: free cholesterol as detected by filipin; GM$_1$: ganglioside GM$_1$ detection utilizing FITC-cholera toxin B subunit.

4 Lipid raft association as defined by 1% TX-100 and sodium carbonate sucrose gradient fractionation methods.

5 Numbers between parentheses refer to the position of palmitoylated cysteines.

6 Weak colocalization noted.

7 ND: Not determined.

8 As determined in (Anderson, 1998; Uittenbogaard *et al.*, 1998; Luetterforst *et al.*, 1999; Luton *et al.*, 1999).

mounting medium were from Molecular Probes. Mouse monoclonal antibodies to lysosome associated membrane protein-1 (LAMP-1) and LAMP-2 were from Developmental Studies Hybridoma Bank (Iowa City, IA). Donkey anti-rabbit IgG-Texas Red (TR) and IgG-FITC, donkey anti-mouse IgG-TR and IgG-FITC secondary Abs, and normal donkey serum (NDS) were obtained from Jackson ImmunoResearch Laboratories. ECL Plus was from Amersham-Pharmacia. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as part of the CellTiter 96 AQ_{ueous} Assay Kit, was from Promega. Paraformaldehyde, filipin, FITC-cholera toxin B subunit (FITC-CTX), methyl β-cyclodextrin (MβCD), water-soluble cholesterol (cholesterol:MβCD complex) and TX-100 were from Sigma. *N*-octyl glucopyranoside (octylglucoside, OG) was from Boehringer Mannheim. All other reagents were of the highest grade commercially available.

3.3.3 Transfection and Immunofluorescence Microscopy

COS-7 cells were cultured and seeded on coverslips as described previously (McCabe and Berthiaume, 1999). All antibodies used were diluted in 4% normal donkey serum (NDS) in PBS to prevent nonspecific binding, and were optimized for fluorophore detection using a Zeiss Laser Scanning Confocal Microscope (model LSM 510) mounted on a Zeiss Axiovert M100 inverted microscope with a 63X plan-apochromatic lens (1.40 NA) (Cross Cancer Institute, University of Alberta). For fluorescent detection of GFP chimeras, mouse anti-GFP (1:200) or rabbit anti-GFP (1:2000) primary Abs were used. Caveolin was detected with rabbit polyclonal anti-caveolin Ab (1:2000). The following antibody dilutions were used: late endosome marker anti-CI-MPR (1:200) (Barr *et al.*, 2000; Shisheva *et al.*, 2001), early endosome marker anti-EEA-1 (1:100) (Sorkina *et al.*, 1999), TGN marker anti-syntaxin 6 (3 μg/ml, 1:80) (Vandenbulcke *et al.*, 2000; Mills *et al.*, 2001), and the late endosome/lysosome markers anti-LAMP-1 and anti-LAMP-2 (1:1000) (Downey *et al.*, 1999). For labeling of the endosomal recycling compartment (ERC), cells were incubated in the presence of 20 μg/ml TR-Tf for 15 min prior to processing to allow for accumulation in this compartment (Teter *et al.*, 1998; Vandenbulcke *et al.*, 2000). All final image analyses were done with Adobe Photoshop 5.5.

3.3.4 Filipin Detection of Cellular Free Cholesterol

Free cholesterol detection in COS-7 cells was achieved using published methods with slight modifications (Cadigan *et al.*, 1990; Mukherjee *et al.*, 1998). Filipin, a fluorescent polyene antibiotic, was used to detect free cholesterol through interactions with a free 3β-hydroxyl group (Muller *et al.*, 1984). Cells were washed four times with PBS and fixed with 1 ml of 2% paraformaldehyde in PBS at room temperature for 1 h, rinsed four times with PBS, and blocked/stained with 1 ml of filipin solution (50 μg/ml) containing 4% NDS in PBS for 1 h at 37°C. The filipin solution was prepared by dissolving 2.5 mg filipin in 1 ml dimethylformamide (DMF), and adding this solution to 49 ml of PBS. When performing colocalization studies, the preceding steps were performed, followed by washes and incubation of the fixed cells with 80 μl primary Ab in 4% NDS in PBS containing 0.1% saponin for 1 h. This was followed by a three PBST (PBS plus 0.1% Tween-20) washes, and secondary Ab steps as above with the addition of 0.1% saponin to the Ab solutions to permeabilize the cells. Addition of 0.1% saponin under these conditions was previously demonstrated not to interfere with cholesterol detection (Cadigan *et al.*, 1990; Abrami and van Der Goot, 1999; Emmerson *et al.*, 2001). Analysis of numerous images of treated cells support the notion that the saponin treatment reduces the cholesterol level at the PM, leaving intracellular cholesterol pools relatively undisturbed. The use of different detergents in this case may affect the cholesterol level in different ways. Confocal microscopic detection of filipin was done with a UV laser 364 nm excitation line and detection with a 385-470 nm bandpass emission filter. Detection of FITC and TR were done using an Argon laser 488 and 568 nm line for excitation, and 505 and 600 nm longpass emission filters, respectively. No bleedthrough was observed between the FITC and UV filipin channels.

3.3.5 Cholesterol Depletion Using Methyl-β–Cyclodextrin

Rapid depletion of cellular cholesterol was performed based on the method of Subtil *et al.* with the following modifications (Subtil *et al.*, 1999). Cells were transfected with plasmids expressing various acylated chimeric GFPs and placed in DMEM containing lipoprotein deficient serum (LPDS) in 6-well plates for 18-20 h to reduce the late endosomal/lysosomal collection of free cholesterol derived from serum lipoproteins (Butler *et al.*, 1992; Mukherjee *et al.*, 1998). Cells were then acutely depleted of cholesterol by incubation for 1 h in 0 and 20 mM of MβCD dissolved in serum-free DMEM at 37°C. Coverslips were then washed, fixed, blocked, and processed for immunofluorescence and cholesterol fluorescent detection. To assess the viability of MβCD-treated cells, a modified single-cell viability assay was used (Racchi *et al.*, 1997). Briefly, transfected COS-7 cells expressing YesGFP were incubated with 20 mM MβCD for 1 h at 37°C. During the last 15 min of the incubation, 100 μl of MTT solution from the CellTiter 96 AQ$_{\text{ueous}}$ Assay Kit was added to 1 ml of media in 6-well dishes, and formazan dye production allowed to develop. To assess recovery after acute cholesterol reduction, transfected cells were incubated with 20 mM MβCD for 1 h at 37°C, followed by incubation of cells with 30 μg/ml of water soluble cholesterol (cholesterol: MβCD complex) for 1 h at 37°C. During the last 15 min, MTT solution was added to detect cell viability. Following these treatments, cells lying on coverslips were processed for immunofluorescence and filipin fluorescence detection as described above. MTT formazan dye produced by viable cells creates initially punctate purple-black crystals observable with a light microscope, and continued or robust production of MTT formazan creates needle-like crystals in viable cells. Experiments were also done on living cells without immunofluorescence processing (*i.e.* live cell GFP detection and MTT formazan observation using epifluorescence and light microscopy), and results obtained were identical to processed samples (**Figure 3.5**). Fixation and permeabilization of cells did not remove MTT formazan crystals produced by viable cells.

3.3.6 GM_1 Glycosphingolipid Detection using FITC-Cholera Toxin B Subunit

COS-7 cells were transfected with various GFP chimeras as described above. Cells were washed with PBS and then incubated for 30 min at 37°C with 2 μg/ml (170 nM) FITC-CTX to label both plasma membrane and intracellular GM_1-containing compartments (Joliot *et al.*, 1997; Harder *et al.*, 1998; Stulnig *et al.*, 1998; Janes *et al.*, 1999). A total of 100 μl of FITC-CTX solution (in 0.1% BSA in serum-free DMEM) was added to each coverslip and immediately placed in a 5% CO_2 incubator at 37°C for 20 min to allow living cell uptake. Following incubation, coverslips were washed four times with serum-free DMEM and processed for normal immunofluorescence as above, with GFP chimeras detected with a TR secondary Ab. Control transfection/fixation experiments were carried out to ensure GFP fluorescence was efficiently quenched under our fixation conditions to prevent bleedthrough into the TR and FITC/filipin channels.

3.3.7 Preparation of Caveolin-Enriched Membrane Fractions Using Sucrose Density Centrifugation

Transfected COS-7 cells grown to near confluence in 100-mm dishes were used to prepare caveolin-enriched membrane fractions. Two established methods, one using the nonionic detergent Triton X-100, and the other a detergent-free method isolating rafts via an increase in pH (sodium carbonate), were used to prepare these fractions (Song *et al.*, 1997; Galbiati *et al.*, 1999a). After two washes with ice-cold PBS, two confluent dishes were scraped into either 2 ml of 500 mM sodium carbonate, pH 11.0, or into 2 ml of MBS (MES-buffered saline, 25 mM MES, pH 6.5, 0.15 M NaCl) containing 1% (w/v) TX-100, and solubilized for 20 min at 4°C. Homogenization was carried out using 10 strokes of a tight-fitting Dounce homogenizer for TX-100-containing samples. For sodium carbonate-treated samples, the cells were homogenized with 10 strokes of a tight-fitting Dounce homogenizer followed by three 10 sec bursts of a Polytron tissue grinder (Brinkmann

Instruments, Westbury, NY, U.S.A.), followed by three 20 sec bursts of sonication (XL Sonicator, Hert Systems, Farmingdale, NY, U.S.A.) combined to more finely disrupt cellular membranes (Song *et al.*, 1996). The homogenates were adjusted to 40% sucrose by addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5-40% discontinuous sucrose gradient was formed above and centrifuged at 39,000 rpm for 16-20 h, in a SW40Ti rotor (Beckmann Instruments, Palo Alto, CA, U.S.A.). A light scattering band was observed at the 5-30% sucrose interface that contained caveolin-rich membranes but excluded most other cellular proteins, as judged by Ponceau S staining of the PVDF membranes. Twelve 1 ml fractions were removed from the top of the tubes, and a portion (32 µl) of each was analyzed by SDS-PAGE analysis followed by Western blot analysis.

3.3.8 In Situ Extraction of Transfected Cells with Non-Ionic Detergent

Separation of cells expressing various acylated GFP constructs into detergent-soluble and detergent–resistant fractions was performed as described with minor modifications (van't Hof and Resh, 1997; Wolven *et al.*, 1997). Confluent 100-mm plates of transfected cells were washed twice with ice-cold STE buffer (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) and then incubated for 20 min with 2 ml of Csk buffer (10 mM Pipes, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 M sucrose, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF) containing 1% TX-100 at 4°C. The resulting detergent-soluble fraction (S) was removed from the cells at different time intervals. The cellular detergent-resistant matrix (R) remaining on the tissue culture dish was incubated with 2.5 ml of ice-cold 1X lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM $MgCl_2$, 5 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF) for 10 min and scraped off the dish with a cell scraper. The content of the soluble fraction was then adjusted with 0.5 ml of 5X lysis buffer. R fractions were balanced with 1X lysis buffer solution and all tubes clarified at 100,000 X g for 15 min at 4°C.

In additional experiments to assess the validity of the lipid rafts isolated in this study, 1% TX-100 in Csk buffer was replaced by a lower TX-100 concentration (0.1%), and 60 mM OG in Csk buffer was used to solubilize raft fractions (Melkonian *et al.*, 1995). Supernatant fractions were removed from the centrifuge tubes without disturbing the pellet, and equivalent portions of both R and S fractions (64 µl) were added to 16 µl of 5X SDS sample buffer containing 100 mM DTT, boiled for 2 min, and analyzed by SDS-PAGE (12.5%). Gels were transferred onto Immobilon-P PVDF membranes (Millipore) (1 h 120 V, 0.5 A). Western blot analyses were performed on these membranes using 1X Blotto as a blocking and diluting solution. Membranes were probed with rabbit anti-GFP (1:2000), anti-Yes monoclonal (1:1000), and rabbit polyclonal anti-caveolin (1:4000) Abs for 1 h at room temperature. HRP-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgGs (1:5000) were used as secondary Abs and incubated with membranes for 1 h at room temperature. 6 x 5 min washes with PBST (PBS plus 0.1% Tween-20) were done between steps. Enhanced chemiluminescence (ECL) detection was performed with the ECL Plus kit (Amersham-Pharmacia Biotech). Detection was from 5 sec to 30 min using Kodak X-OMAT AR film. Control experiments were conducted to ensure no crossreactivity between the anti-caveolin, anti-Yes, and anti-GFP Abs was occurring. Membranes were stained post-ECL detection with Coomassie Brilliant Blue to demonstrate reproducibility, the relative distributions of cellular proteins, and the alterations in these distributions caused by detergent extraction.

3.4 RESULTS

3.4.1 Colocalization of Acylated Chimeric GFPs with Free Cholesterol

Following the determination of the subcellular localization of N-terminally acylated GFP chimeras (McCabe and Berthiaume, 1999), we sought to further characterize the mechanisms leading to acylation-specific subcellular localization. In our previous study, we demonstrated that localization of myristoylated and variably palmitoylated chimeric GFPs was independent of amino acid sequence surrounding the dually acylated myr-GlyCys N-terminal motif. Therefore, we postulated that biophysical properties of both the acylated chimeric GFPs and the acceptor membranes would be at play in conferring proper localization. Lipid rafts/DRMs/caveolae are known to be enriched in cholesterol, glycosphingolipids, saturated glycerolipids and caveolin. Since several N-terminally acylated signaling proteins have been found to be associated with these membrane domains (Kurzchalia and Parton, 1999), the rationale behind our investigation was to characterize the potential involvement of constituents of these lipid rafts in the location of our chimeric GFPs to a variety of membranes.

In order to evaluate the cholesterol requirements of the acceptor membranes, we localized intracellular pools of free cholesterol using an established procedure using filipin (Cadigan *et al.*, 1990). We also altered the cholesterol content of COS-7 cells using the efficient cholesterol-depleting agent, MβCD. When fixed, transfected cells were stained with filipin (**Figure 3.1**), we found that myristoylated plus palmitoylated YesGFP and the filipin-derived free cholesterol signal showed very similar distributions and localization at the plasma membrane (arrowheads), and in vesicular structures previously identified as endosomes (arrows) (McCabe and Berthiaume, 1999). This pattern of plasma membrane and endosomal localization of dually acylated GFPs and cholesterol was consistent for all chimeric GFP constructs containing myristate and palmitate (YesGFP, $G\alpha_o$GFP, and LckGFP), despite the fact that these constructs vary in the number of palmitoylated cysteines and their position in the primary sequence (**Table 3.1** and **Figure 3.2**). Similarities in distribution and localization of signals corresponding to myristoylated nonpalmitoylated Yes(C3S)GFP or Fyn(C3,6S)GFP and filipin were also found (**Figure 3.2**). Signals corresponding to cytosolic nonacylated constructs, such as Yes(G2A)GFP and GFP alone, did not overlap with those of filipin (**Figure 3.1**). Myristoylated and polybasic region-containing Src_{16}GFP also demonstrated plasma membrane (arrowheads) and endosomal (arrows) colocalization with filipin. Dually palmitoylated (nonmyristoylated) GAP-43GFP colocalized with filipin at the plasma membrane (arrowheads) and in a dense focal perinuclear area (arrow). This perinuclear area was previously identified as the Golgi apparatus based on giantin colocalization (McCabe and Berthiaume, 1999). Apparent colocalization between the weak endogenous $p62^{c\text{-}yes}$ signal and filipin was also demonstrated (**Figure 3.1**).

3.4.2 Cholesterol Depletion Affects Acylated GFP and Cholesterol Distribution

When we assessed the effects of cholesterol depletion on the structures harboring acylated GFPs, we found a significant relocalization of the fatty acylated GFPs as well as the sphingolipid GM_1. As shown in **Figure 3.3A**, in the absence of MβCD, YesGFP was found at the plasma membrane, on peripheral endosomes as well as clustered in a focal perinuclear area. Free cholesterol, as detected by filipin fluorescence, was seen concentrated in endosomal structures and at the plasma membrane. Colocalization of cholesterol and YesGFP in endosomal structures (arrows) and at the plasma membrane (arrowhead) was evident. In cells treated with 20 mM MβCD, a marked redistribution of YesGFP signal was seen reproducibly. The chimeric GFP signal appeared to be more concentrated at the plasma membrane of treated cells. The distribution of the fluorescent signal corresponding to GFP was also more homogeneous (less punctate) in treated versus

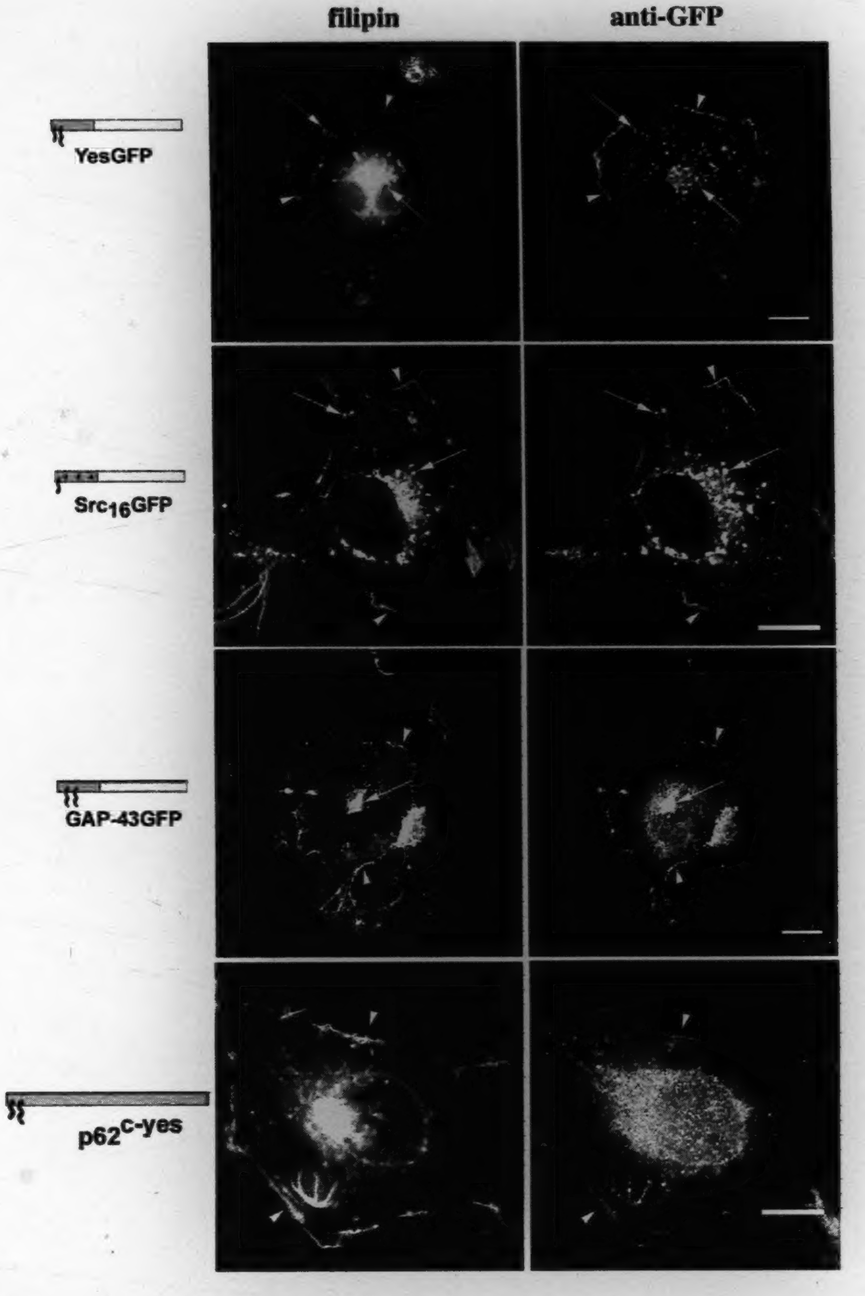

Figure 3.1. Colocalization of chimeric GFPs and p62$^{c\text{-}yes}$ with free cholesterol in COS-7 cells. Cells expressing various GFP chimeras were fixed, permeabilized, and incubated with filipin to detect free cholesterol, rabbit anti-GFP Ab followed by FITC-conjugated anti-rabbit Ab to detect acylated GFPs, and mouse anti-Yes PTK followed by FITC-conjugated anti-mouse Ab to detect endogenous p62$^{c\text{-}yes}$. Arrowheads highlight areas of plasma membrane colocalization, and arrows highlight endosomal/Golgi colocalization. Bars, 10 μm.

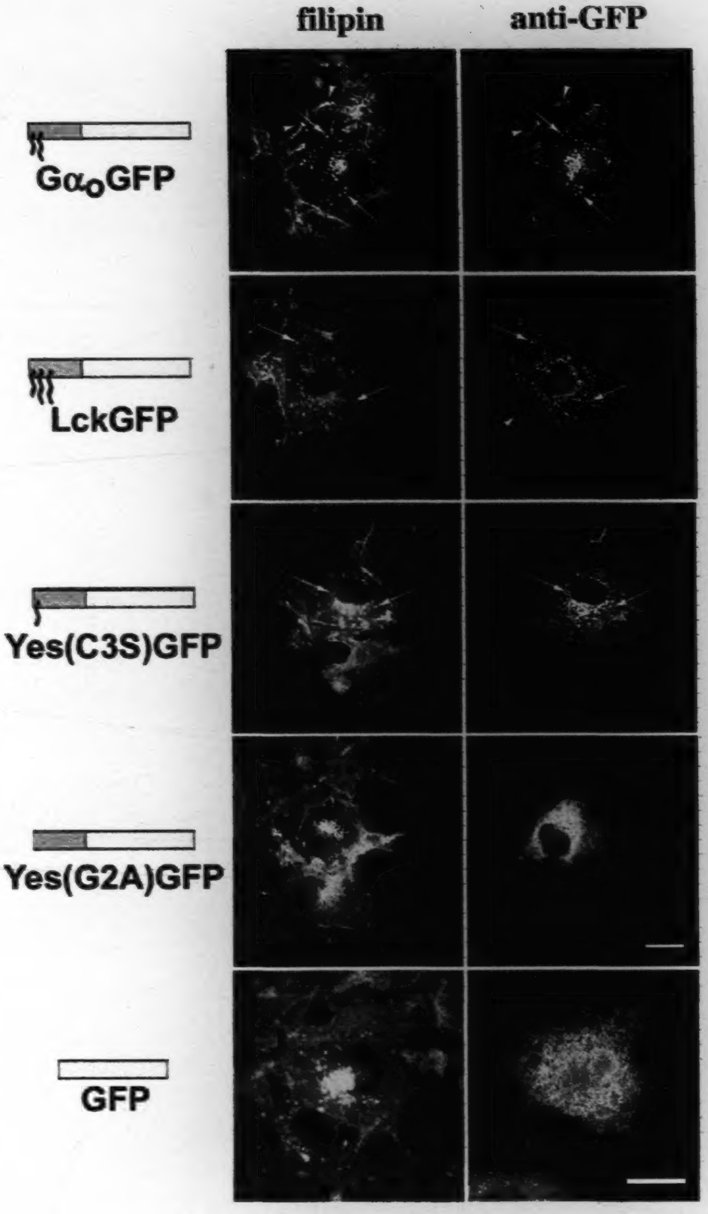
filipin
anti-GFP
Gα_oGFP
LckGFP
Yes(C3S)GFP
Yes(G2A)GFP
GFP

Figure 3.2. Colocalization of chimeric GFPs with free cholesterol in COS-7 cells. Cells transfected with plasmids expressing various GFP chimeras were fixed, permeabilized, and incubated with filipin to detect free cholesterol, and rabbit anti-GFP Ab followed by FITC-conjugated anti-rabbit Ab to detect acylated GFPs. Arrowheads highlight areas of plasma membrane colocalization, and arrows highlight endosomal/Golgi colocalization. Bars, 10 μm.

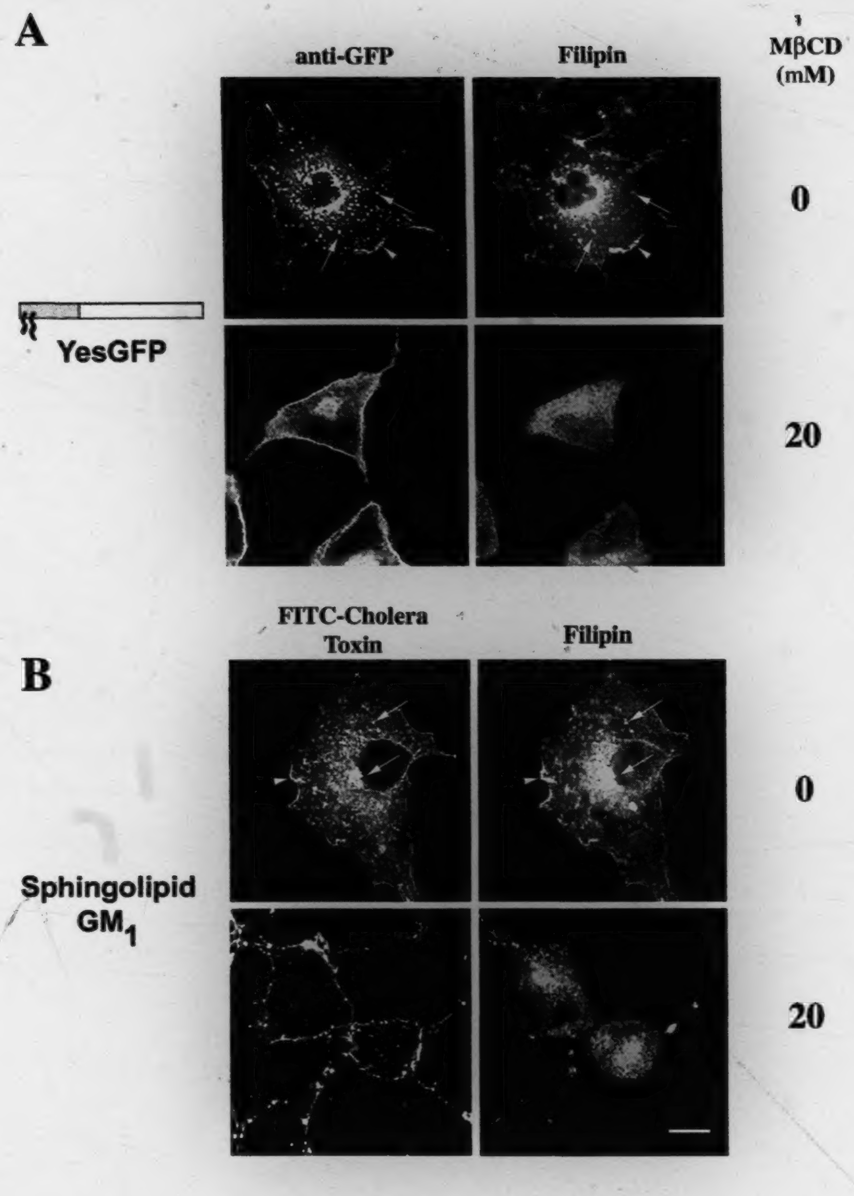
A
anti-GFP
Filipin
MβCD (mM)
0
YesGFP
20
B
FITC-Cholera Toxin
Filipin
0
Sphingolipid GM1
20

Figure 3.3. Acute cholesterol depletion alters intracellular localization of acylated GFP chimeras and GM$_1$. COS-7 cells, grown in lipoprotein deficient serum for 18-20 h, were acutely depleted of cholesterol using 20 mM MβCD for 1 h at 37°C. (A), GFP chimeras were detected using rabbit anti-GFP and FITC-conjugated donkey anti-rabbit secondary Ab. Free cholesterol was detected with filipin. (B), ganglioside GM$_1$ distribution detected with FITC-CTX. Arrowheads highlight areas of plasma membrane colocalization, and arrows highlight endosomal colocalization. Bar, 10 μm.

control cells. Acylated GFPs were also found clustered in a perinuclear organelle, and a striking reduction in punctate endosomal structures normally decorated by myristoylated and palmitoylated GFPs was evident. Cells treated with MβCD showed a significant reduction in size consistent with a previous report (Ilangumaran and Hoessli, 1998). The efficiency of our MβCD treatment in free cholesterol removal can be appreciated by the reduced intensity of the filipin signal when cells were observed under identical conditions as compared to control cells. Our experimental conditions utilize concentrations of MβCD known to be efficient in cholesterol removal in COS-7 and other cells. The use of 10 mM MβCD over 30 min has been shown to cause a 50% reduction in quantifiable free cholesterol in CHO cells (Subtil *et al.*, 1999), and 5 mM MβCD reduced free cholesterol to 64% of control over 2 h in COS-7 cells (Racchi *et al.*, 1997). Results presented in **Figure 3.3A** using YesGFP are typical of all acylated chimeric GFPs (FynGFP, LckGFP, $G\alpha_o$GFP, GAP-43GFP, and Src_{16}GFP) (**Figure 3.4**).

To assess the effects of cholesterol depletion on another constituent of lipid rafts/DRMs, the ganglioside GM_1, COS-7 cells were incubated in the presence of MβCD as described above and FITC-CTX was utilized to detect the ganglioside. As can be seen in **Figure 3.3B**, in the absence of MβCD, the FITC signal corresponding to GM_1 was detected at the plasma membrane (arrowhead), and in punctate structures clustered in a perinuclear area and in the periphery (arrows). When compared to the distribution of the filipin signal, extensive similarity was observed and significant colocalization was evident at the plasma membrane (arrowhead), and in intracellular endosomal structures (arrows). In contrast, when cells were treated with 20 mM MβCD, a significant alteration in the GM_1 distribution was observed. In the vast majority of cells, essentially no intracellular vesicular structures were visible, and the plasma membrane GM_1 fraction became more prominent and displayed relatively large areas of GM_1 clustering when compared to untreated cells.

To assess the effects of MβCD treatment on cellular viability, we utilized the tetrazolium dye, MTT, a well established marker of cellular viability. As can be seen in **Figure 3.5**, treatment with 20 mM MβCD for 1 h at 37°C caused changes in cell morphology, altered distribution of acylated GFP chimeras, reduced the apparent number of endocytic structures but MTT formazan crystals were still produced by treated cells albeit at a lower level on average. Upon treatment with 20 mM MβCD followed by replenishment of cholesterol with 30 μg/ml cholesterol:MβCD complex, a restoration of more normal cellular morphology occurred, along with the distribution of acylated GFP-containing to peripheral endocytic structures. In addition, robust production of MTT formazan crystals was seen in cholesterol-replenished cells, indicating apparently normal cellular function. Thus, acute cholesterol depletion did not affect cellular viability under these conditions, and

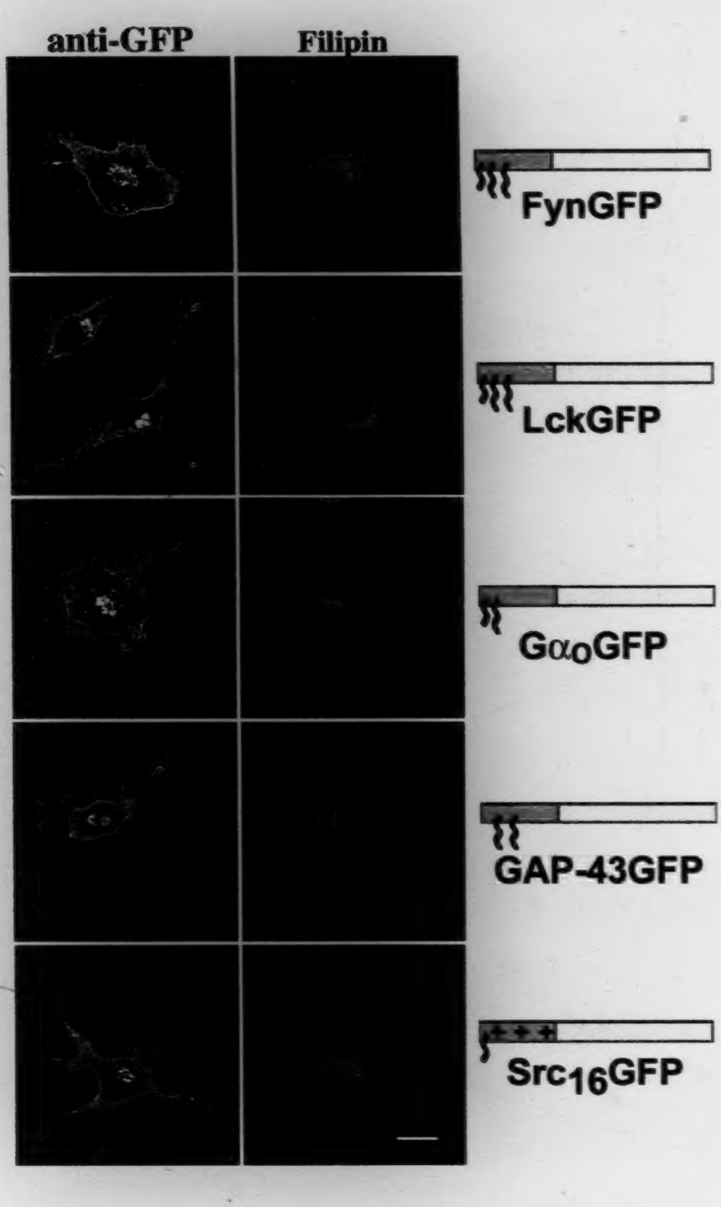
anti-GFP
Filipin
FynGFP
LckGFP
GαoGFP
GAP-43GFP
+ + +
Src16GFP

Figure 3.4. Acute cholesterol depletion alters intracellular localization of acylated GFP chimeras. GFP chimeras were detected using rabbit anti-GFP and FITC-conjugated donkey anti-rabbit secondary Ab. Free cholesterol was detected with filipin. Bar, 10 μm.

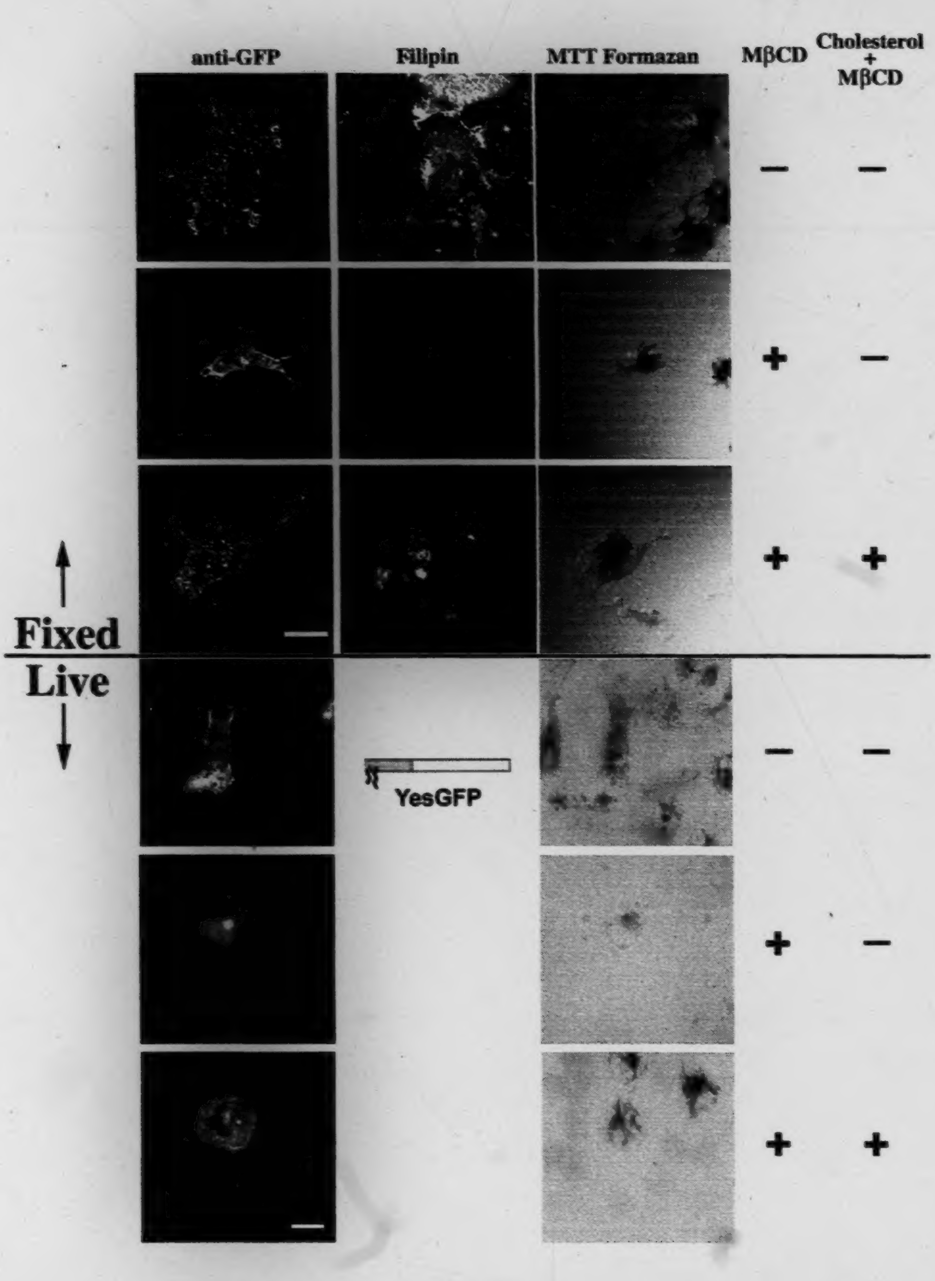
anti-GFP
Filipin
MTT Formazan
MβCD
Cholesterol + MβCD
− −
+ −
+ +
Fixed
Live
− −
YesGFP
+ −
+ +

Figure 3.5. Viability of MβCD–treated COS-7 cells expressing YesGFP. Expressing cells were treated with vehicle, 20 mM MβCD for 1 h at 37°C, or with 20 mM MβCD for 1 h followed by repletion with 30 μg/ml cholesterol:MβCD for 1 h at 37°C. Cellular viability was determined by MTT formazan production in both fixed cells (upper panels) or living cells (lower panels). GFP chimeras were detected using rabbit anti-GFP and FITC-conjugated donkey anti-rabbit secondary Ab. Free cholesterol was detected with filipin. Bars, 10 μm.

was completely reversible with supplementation of exogenous free cholesterol. Experiments were done in living and fixed COS-7 cells, showing identical results. In addition, cholesterol-replenished cells excluded Trypan Blue dye, another indicator of cellular viability.

Attempts to lower cellular sphingolipids with the inhibitor of ceramide synthase, fumonisin B_1 were carried out. Due to the toxicity and length of the treatment to achieve significant reduction in cellular sphingolipids, we were unfortunately unable to photograph viable cells with satisfactory results using transiently transfected cells.

3.4.3 Colocalization of Acylated Chimeric GFPs with Organelle Markers

Acylated GFP chimeras have been previously shown to associate with intracellular structures identified as endosomes based on colocalization with the fluorescent lipoprotein particle, DiI-LDL, internalized via receptor-mediated endocytosis (McCabe and Berthiaume, 1999). To further assess the type of endocytic organelles where acylated chimeric GFPs are localized in comparison to free cholesterol localization, we utilized COS-7 established organelle markers in indirect triple immunofluorescence/filipin fluorescence protocols. Myristoylated/palmitoylated YesGFP and myristoylated/polybasic region-containing Src_{16}GFP colocalized significantly with the early endosomal marker EEA1 (**Figure 3.6**), and with the ERC marker TR-Tf and the late endosomal/lysosomal marker LAMP-2 (results not shown). Only partial colocalization was seen between YesGFP or Src_{16}GFP and the TGN marker, syntaxin 6 (results not shown). Similarly, little or no colocalization was seen between these two chimeric GFPs and the TGN/late endosomal marker CI-MPR (**Figure 3.6**). In contrast, the dually palmitoylated, nonmyristoylated GAP-43GFP showed little or very partial colocalization with EEA1 (**Figure 3.6**) and TR-Tf or with LAMP-2 (results not shown), but demonstrated significant colocalization with CI-MPR (**Figure 3.6**) and syntaxin 6 (results not shown). Thus, differentially acylated chimeric GFPs are found enriched in different endocytic organelle compartments under steady state. The specificity of association depends on the type of N-terminal acylation signal present in the GFP chimera. The corresponding free cholesterol distributions are shown below the merged immunofluorescence images in grayscale, and demonstrate highly similar signal distributions between filipin/free cholesterol and the organelle markers studied in **Figure 3.6**. These results are suggestive but not conclusive that the GFP chimeras are in rafts. Due to the limitation of resolution of the confocal microscope, and the rough estimate of raft size being quite small (20-400 nm), it is possible that the GFP chimeras are not in rafts proper.

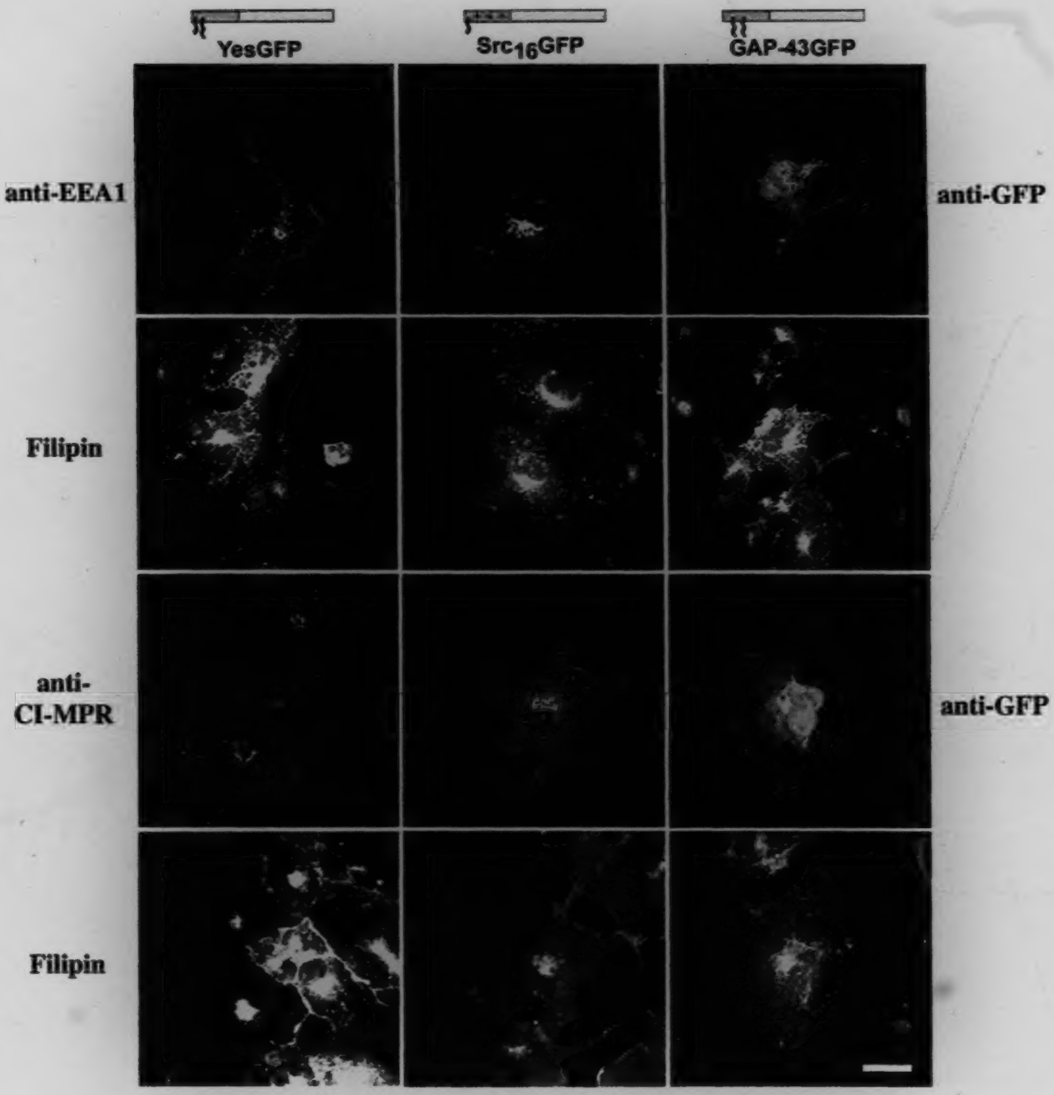
YesGFP
Src16GFP
GAP-43GFP
anti-EEA1
anti-GFP
Filipin
anti-
CI-MPR
anti-GFP
Filipin

Figure 3.6. Triple colocalization of various chimeric GFPs with early endosome and TGN organelle markers and free cholesterol as detected by filipin in COS-7 cells. Colocalization of differentially acylated GFP chimeras (green) and organelle marker (red) distributions in transfected COS-7 cells can be seen in color. Filipin signal is shown in grayscale panels below corresponding color organelle colocalization panels. Bar, 10 μm.

3.4.4 Acylated Chimeric GFPs Colocalize with the Ganglioside GM_1

The ganglioside GM_1 is a known marker of caveolae, but can also be found in other membrane domains (Parton, 1994; Iwabuchi *et al.*, 1998). To compare the distribution of GM_1 to that of our acylated GFP chimeras, COS-7 cells were fixed and permeabilized to abolish mature GFP epifluorescence (Pines, 1995), and FITC-CTX was utilized to detect GM_1. Numerous reports regarding the usage of chromophore-conjugated CTX B subunit to detect GM_1 exist (Joliot *et al.*, 1997; Harder *et al.*, 1998; Orlandi and Fishman, 1998; Janes *et al.*, 1999). GFP was detected using our rabbit polyclonal antibody followed by incubation with donkey anti-rabbit TR-conjugated secondary Ab (thus in **Figure 3.7A**, GFP staining is shown in red). Appropriate controls were done at the time of each experiment to ensure total inactivation of intrinsic GFP epifluorescence during our fixation conditions (**Figure 3.7B**).

We analyzed transfected COS-7 cells to determine if our acylated chimeras would co-localize with GM_1. As shown in **Figure 3.7A**, similar distributions of the ganglioside GM_1 and YesGFP were found in endosomes (arrows) and at the plasma membrane (arrowheads). Myristoylated Src_{16}GFP, containing a polybasic membrane-associating signal, also displayed significant colocalization with GM_1 in endosomes (arrows) and at the plasma membrane (arrowhead). In contrast to the above patterns of colocalization, dually palmitoylated GAP-43GFP, colocalized with GM_1 in the Golgi/TGN (arrow) and at the plasma membrane (arrowhead), but not in peripheral endosomes. The distribution of endocytosed FITC-CTX (and hence GM_1) has been recently assessed in COS-7 cells (Nichols *et al.*, 2001), and found to accumulate in transferrin-labeled compartments as well as the Golgi complex. This was previously shown for free cholesterol (Mukherjee *et al.*, 1998). Thus, our results demonstrating the colocalization of acylated GFP chimeras, cholesterol, and GM_1 in these compartments appear to be valid. The distributions of caveolin and the ganglioside GM_1 overlapped at the plasma membrane and focal perinuclear structures, but this overlap was only partial (**Figure 3.7C**).

3.4.5 Most Acylated Chimeric GFPs do not Colocalize with Caveolin

Several acylated signaling proteins have been documented to be present in caveolae (Oh and Schnitzer, 1999). To assess whether caveolin and acylated GFPs colocalized, we utilized indirect double immunofluorescence. As shown in **Figure 3.8**, the signals for acylated YesGFP and Src_{16}GFP, when merged with the caveolin signal, displayed minimal colocalization, especially in peripheral endosomal vesicles. In the tightly clustered Golgi and endosomal recycling compartment area (arrows), some colocalization is apparent

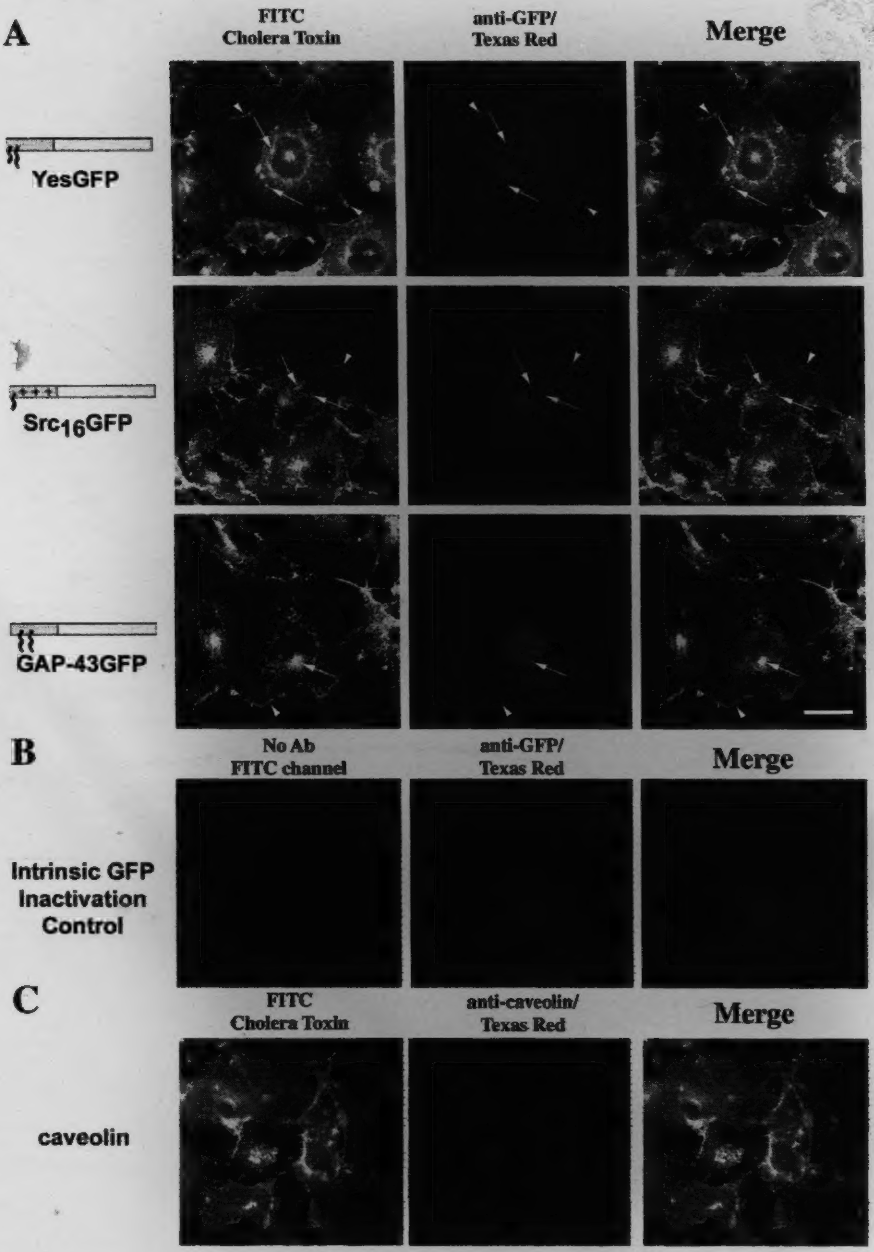
A
FITC
Cholera Toxin
anti-GFP/
Texas Red
Merge
YesGFP
Src16GFP
GAP-43GFP
B
No Ab
FITC channel
anti-GFP/
Texas Red
Merge
Intrinsic GFP
Inactivation
Control
C
FITC
Cholera Toxin
anti-caveolin/
Texas Red
Merge
caveolin

Figure 3.7. Colocalization of chimeric GFPs with GM$_1$. (A), colocalization of GM$_1$ as detected with FITC-CTX (green) with acylated chimeric GFPs YesGFP, Src$_{16}$GFP, and GAP-43GFP, as detected using rabbit anti-GFP Ab followed by TR–conjugated donkey anti-rabbit Ab. Arrowheads highlight areas of plasma membrane colocalization, and arrows highlight endosomal/Golgi colocalization. (B), panel of images demonstrating inactivation of intrinsic GFP fluorescence, when detected with TR-conjugated secondary Ab. (C), micrographs depicting partial caveolin-GM$_1$ colocalization. Bars, 10 µm.

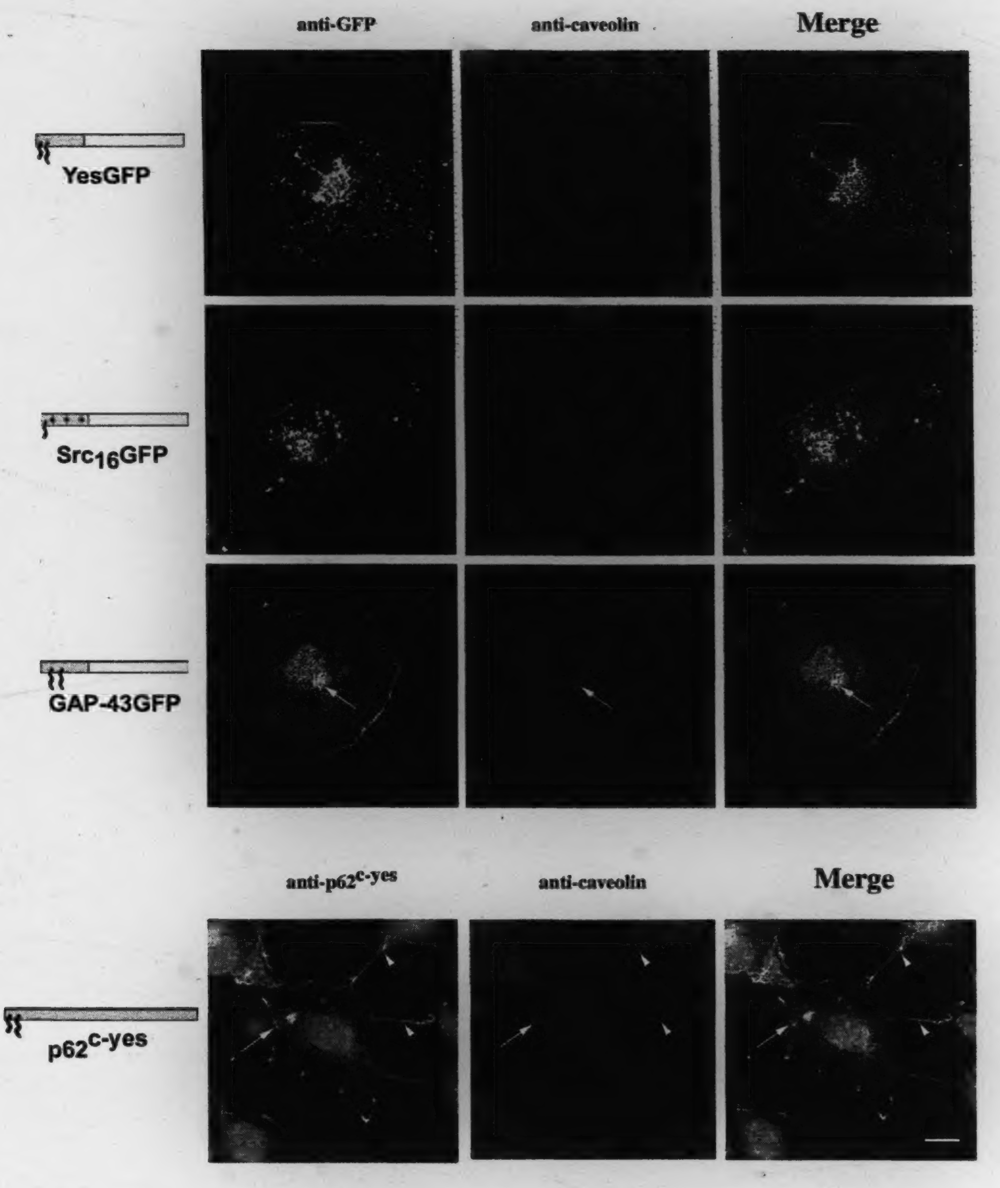
anti-GFP
anti-caveolin
Merge
YesGFP
Src16GFP
GAP-43GFP
anti-p62c-yes
anti-caveolin
Merge
p62c-yes

Figure 3.8. Colocalization of chimeric GFPs and endogenous $p62^{c\text{-}yes}$ PTK with caveolin. The GFP chimeras were detected by incubation with mouse anti-GFP Ab followed by FITC-conjugated anti-mouse Ab. $p62^{c\text{-}yes}$ was detected with mouse anti-Yes Ab, and FITC-conjugated donkey anti-mouse secondary Ab. Arrows point to caveolin-GAP-43GFP Golgi colocalization. In the lower panel, arrowheads point to colocalization of acylated GFP-containing structures and caveolin-containing structures in cell-cell contacts, and intracellularly (arrow). Bars, 10 μm.

(yellow), but may represent the overlap of two intense signals in proximity of one another. In contrast to these results, the dually palmitoylated GAP-43GFP displayed significant colocalization with caveolin in a perinuclear focal compartment (**Figure 3.8**, arrow) previously identified as the Golgi apparatus. GAP-43GFP did not colocalize with caveolin significantly at any other sites. The distribution of endogenous p62$^{c\text{-}yes}$ was also compared with that of caveolin. When merged with the caveolin signal, minor colocalization was found intracellularly (arrow), and at cell-cell contacts (arrowheads).

3.4.6 Acylated GFPs do not Cofractionate with Caveolin-Enriched Lipid Rafts – but Full Length p62$^{c\text{-}yes}$ Tyrosine Kinase Does

The presence of acylated signaling proteins in caveolae or lipid rafts has, in the past, been assessed using full-length signaling proteins with or without their acylation sites removed. We were interested in performing the converse experiment in order to assess if addition of lipid anchors to a reporter protein could confer detergent resistance (enrichment in lipid rafts) to that reporter protein. To do so, COS-7 cells expressing various chimeric GFPs were grown to confluence in 100 mm dishes and used to prepare caveolin-enriched lipid rafts, using two different established methods. Using a detergent-based isolation procedure (Song *et al.*, 1997), in contrast to the presence of caveolin at the 5-30% sucrose interface (fractions 4 and 5), all GFP chimeras tested and GFP alone were present in the non-buoyant fractions 9 to 12. In contrast, full-length p62$^{c\text{-}yes}$ did cofractionate with caveolin in buoyant DRMs (**Figure 3.9A**). Using a cold 1% TX-100 sucrose density gradient fractionation method followed by Western blot analysis of aliquots for caveolin, we showed that caveolin was clearly enriched in the low density raft fractions (lanes 4-5, at the 5-30% interface). In addition, caveolin was also present in higher density detergent-soluble fractions as well. This has also been seen by others in COS cells (Joliot *et al.*, 1997). Overexposed blots did not show any chimeric GFP signal in the lipid raft region of the membrane. Coomassie-stained PVDF membranes demonstrated the presence of the vast majority of cellular proteins in the TX-100 soluble 40% sucrose fractions (**Figure 3.10**). When pellets found at the bottom of the ultracentrifuge tube were analyzed for chimeric GFPs or caveolin, only a small residual amount of both proteins was found, and likely represented trapped molecules present in large cytoskeletal aggregates (**Figure 3.10**).

In order to avoid possible detergent-induced artifacts in the preparation of lipid rafts, we prepared lipid rafts using an established detergent-free lipid raft isolation procedure (Song *et al.*, 1997). As seen in **Figure 3.9B**, YesGFP, Yes(C3S)GFP, Yes(G2A)GFP, FynGFP, Gα_oGFP, GAP-43GFP and GFP alone did not cofractionate

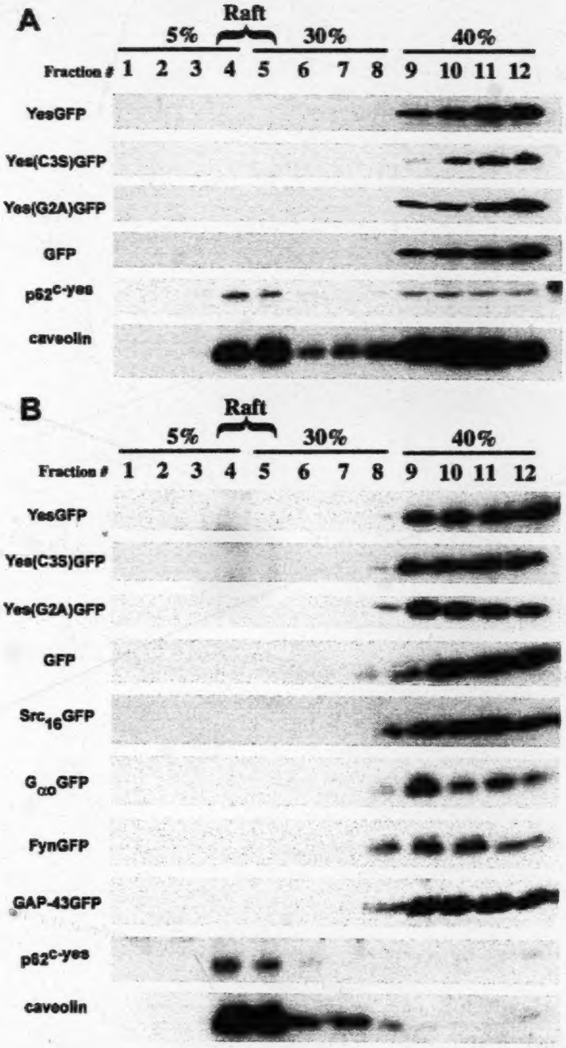
A
Raft
5%
30%
40%
Fraction # 1 2 3 4 5 6 7 8 9 10 11 12
YesGFP
Yes(C3S)GFP
Yes(G2A)GFP
GFP
p62c-yes
caveolin
B
Raft
5%
30%
40%
Fraction # 1 2 3 4 5 6 7 8 9 10 11 12
YesGFP
Yes(C3S)GFP
Yes(G2A)GFP
GFP
Src16GFP
GαoGFP
FynGFP
GAP-43GFP
p62c-yes
caveolin

Figure 3.9. Caveolin-enriched lipid raft isolation using detergent-based and detergent-free sucrose density gradient fractionation methods. (A) caveolin-enriched lipid raft isolation using a TX-100 at 4°C fractionation method. COS-7 cells transiently transfected with various acylated GFPs and GFP alone were subjected to subcellular fractionation after homogenization in buffer containing 1% TX-100. The distribution of acylated chimeric GFPs and endogenous caveolin are shown using a discontinuous 5-40% sucrose gradient. Fractions were collected from the top of the gradient, separated by SDS-PAGE (12.5% acrylamide), and analyzed by immunoblotting and Coomassie staining of the immunoblot. Immunoblot analysis was done with rabbit anti-GFP Ab (1:2000) to detect GFP and the acylated chimeras, with anti-caveolin (1:4000) to detect endogenous caveolin, and with mouse anti-Yes Ab (1:1000) to detect endogenous $p62^{c\text{-}yes}$ PTK. (B) caveolin-enriched lipid raft isolation using a sodium carbonate fractionation method. Transfected COS-7 cells were subjected to subcellular fractionation after homogenization in buffer containing 500 mM sodium carbonate, pH 11.0. The distribution of acylated chimeric GFPs and endogenous caveolin and $p62^{c\text{-}yes}$ PTK are shown. Fractions were collected and analyzed as above.

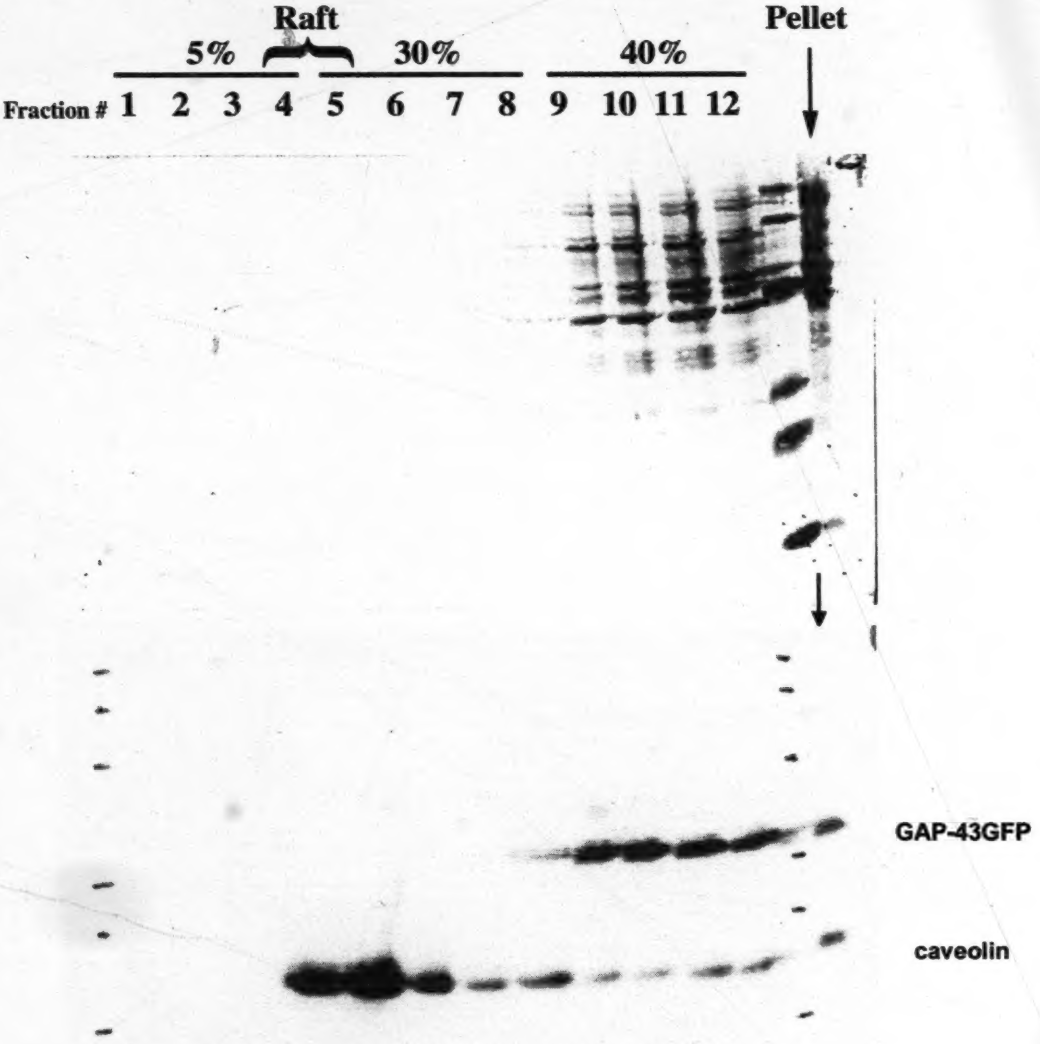

Figure 3.10. Distribution of cellular proteins in sucrose density gradient raft and pellet fractions. The upper panel shows a typical Coomassie-stained PVDF membrane, with the vast majority of cellular proteins found within the high-density 40% sucrose fractions (9-12). The contents of the post-centrifugation pellet can be seen at far right (arrow). The lower panel shows an immunoblot analysis with rabbit anti-GFP Ab (1:2000) to detect GFP and the acylated chimeras, with anti-caveolin (1:4000) to detect endogenous caveolin. Small residual amounts of both proteins are found in this fraction (arrow), likely representing trapped molecules in large cytoskeletal aggregates.

with caveolin in the buoyant DRM fractions 4-5. Of note, the detergent-free sodium carbonate method led to a much more restricted distribution of caveolin signal, with fractions 4-5 containing the majority of caveolin signal, and minor amounts in fractions 6-8. To assess whether full-length Yes PTK also floated into raft membranes using this detergent-free method, membranes were also blotted with monoclonal anti-Yes Ab. Full-length Yes PTK also cofractionated with caveolin-rich membranes fractions containing lipid rafts (4 and 5). We conclusively show that dual acylation and acylation combined with a polybasic domain are not sufficient to confer lipid raft localization, independent of the isolation procedure.

3.4.7 Acylated GFPs Are Readily Detergent Soluble

When transfected COS-7 cells were fractionated into S100 and P100 fractions in the absence of detergent, we found more than 90% of dually acylated chimeric GFPs in the P100 fraction (McCabe and Berthiaume, 1999). To further assess the membrane fractionation properties of the acylated chimeric GFPs analyzed in this study, a second procedure allowing separation of detergent-soluble and -resistant membrane fractions was utilized. Using a method that allows rapid cellular fractionation and separation of crude S100 "soluble" and P100 "pellet" fractions, we compared the solubilization of our chimeric GFPs and caveolin in a variety of detergent conditions (van't Hof and Resh, 1997). First, transiently transfected COS-7 cells expressing different chimeric GFP cDNAs were extracted with either 1% TX-100, 0.1% TX-100, or 60 mM OG at 4°C for 20 min. The constructs analyzed in this experiment included a member of each of the three membrane association signal combinations (*i.e.*, YesGFP, Src_{16}GFP, and GAP-43GFP). After 20 min of extraction, all chimeric GFPs were found exclusively in the TX-100 soluble fraction (**Figure 3.11A**). Thus, the presence of myristate and/or palmitate moieties with or without adjacent polybasic domains had no bearing on the detergent solubility in these experiments. In contrast to these results, the distribution of caveolin between soluble (S) and resistant pellet (P) fractions was markedly different. Caveolin was mostly found (~60%) or largely found (80-90%) in the pellet fractions in the presence of 1% TX-100 or 0.1% TX-100, respectively. In contrast, and in agreement with previous reports, caveolin was readily solubilized in the presence of 60 mM OG (Melkonian *et al.*, 1995). Coomassie-stained PVDF membranes demonstrate the efficiency of detergent extractions (0.1 or 1% TX-100, and 60 mM OG) which led to a near complete solubilization of total cellular proteins (**Figure 3.11B**).

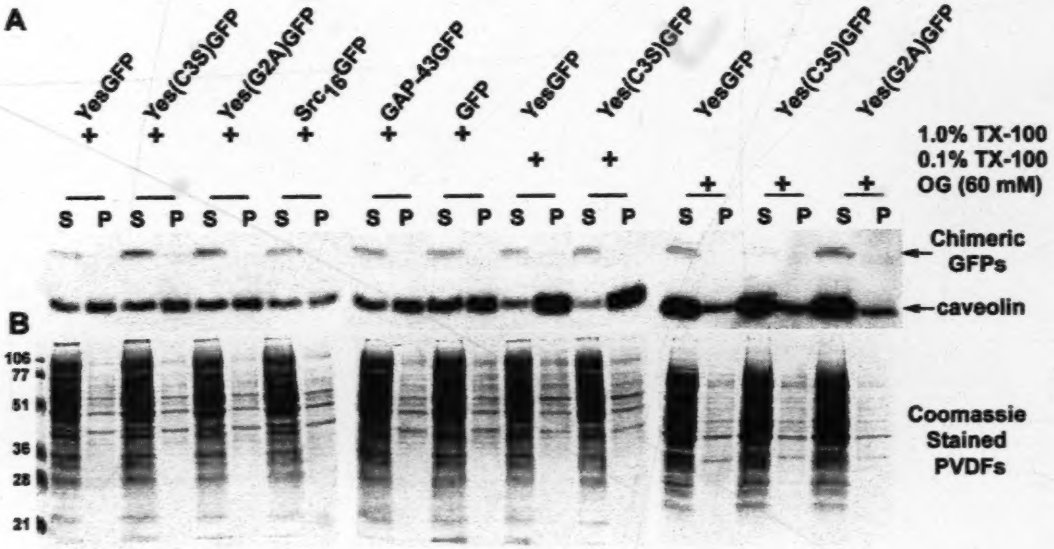
A
YesGFP
Yes(C3S)GFP
Yes(G2A)GFP
Src16GFP
GAP-43GFP
GFP
YesGFP
Yes(C3S)GFP
YesGFP
Yes(C3S)GFP
Yes(G2A)GFP
1.0% TX-100
0.1% TX-100
OG (60 mM)
+
S
P
Chimeric GFPs
caveolin
B
106
77
51
36
28
21
Coomassie Stained PVDFs

Figure 3.11. Solubilization of acylated chimeric GFPs by detergent at 4°C. COS-7 cells expressing various acylated and nonacylated GFP chimeras, and GFP alone, were solubilized in 0.1 or 1% TX-100 and 60 mM OG at 4°C for 20 min, and then separated into soluble (S) and resistant (P) fractions. Aliquots of soluble and resistant fractions were analyzed by immunoblotting using rabbit anti-GFP and anti-caveolin Abs. (A) immunoblots show the distribution of the expressed proteins and endogenous caveolin in soluble (S) and resistant (P) fractions in the presence of indicated detergents. (+) signs indicate the type and concentration of detergent used to solubilize the cells. (B) Coomassie stained PVDFs, corresponding to the immunoblots above, depicts distribution of total cellular protein after detergent solubilization.

3.4.8 Acylated GFPs, but not Caveolin and p62$^{c\text{-}yes}$, are Rapidly Solubilized from Cell Membranes

To further assess whether the various fatty acylated chimeric GFPs were present or not in DRMs, and whether their presence in DRMs would be dependent on the time exposure of membranes to TX-100 detergent, we incubated cells expressing chimeric GFPs in the presence of a 1% TX-100 solution at 4°C for various time intervals (from 0 to 20 min). As shown in **Figure 3.12A**, at 0 min, essentially all GFP constructs, caveolin, and p62$^{c\text{-}yes}$ were found in the TX-100 resistant ("P") fraction. All constructs tested (YesGFP, Src$_{16}$GFP, and GAP-43GFP) showed a time-dependent solubilization which was essentially complete by 20 min. All constructs showed similar time-dependent extraction kinetics, suggesting that solubilization of acylated GFPs is very rapid, and that all acylated GFPs are found in membrane domains that are TX-100 soluble at 4°C. Full-length caveolin (24 kDa) and Yes PTK (62 kDa) proteins were present on the PVDF membranes at their expected molecular weights, were much more resistant to TX-100 extraction, and were found largely in the pellet fraction. Coomassie-stained PVDFs below the Western blots are shown to demonstrate the reproducibility and efficiency of the extraction/solubilization procedure. Finally, in **Figure 3.12B**, the specificity of the three antibodies used in this study is shown using aliquots of a fractionated cell lysate of transiently transfected COS-7 cells expressing YesGFP. The lysate was prepared using 1% TX-100 at 4°C with 20 min incubation as described above. In additional controls, Abs were also combined pairwise and unequivocally demonstrated that no crossreactivity was occurring between the Abs (**Figure 3.12C**).

3.5 DISCUSSION

In order to investigate the mechanisms of lipidation-dependent subcellular localization, we analyzed the biochemical components and properties of both acylated proteins and acceptor membranes. We show that addition of a dual palmitoylation signal or N-terminal myristoylation signal combined with either a palmitoylation signal or polybasic region onto a GFP reporter protein conferred detergent-soluble localization to membranes enriched in free cholesterol and sphingolipids. Our data suggest that protein fatty acylation of an otherwise cytosolic reporter protein is not sufficient to confer fractionation to detergent resistant membrane or partitioning to lipid rafts. Likely protein-protein interactions are required for these processes to occur. Overall, our data are consistent with the possibility that fatty acylated GFPs are only loosely associated with lipid rafts and that multiple types of rafts exist inside the cell, caveolae representing only one of them.

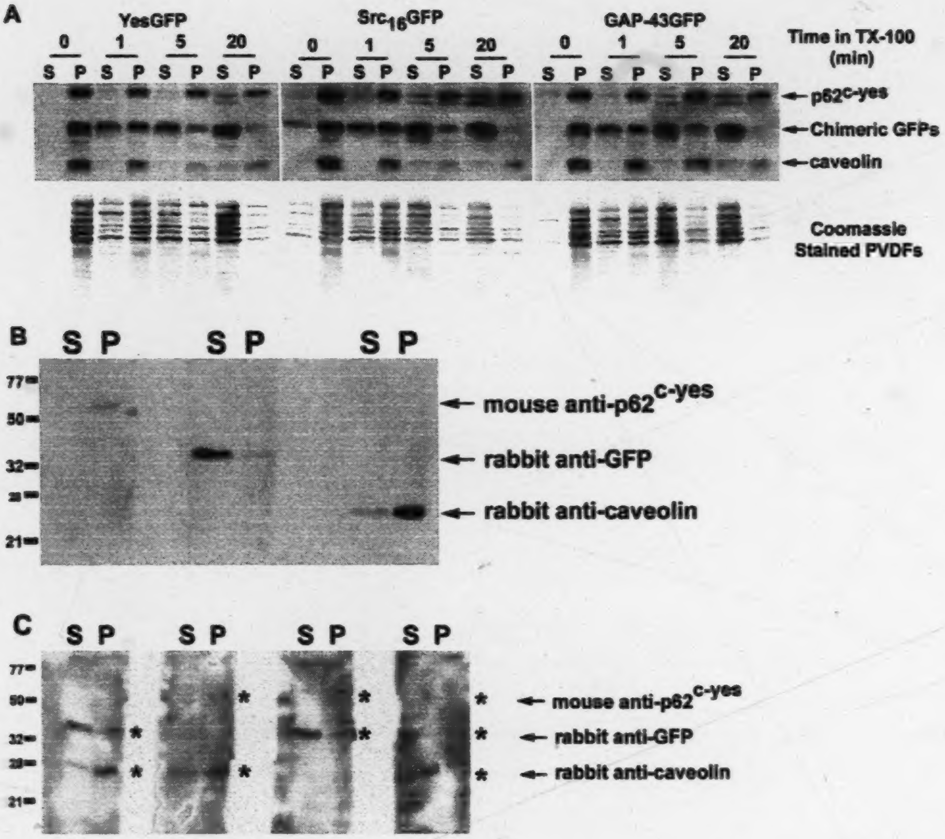
A
YesGFP
Src16GFP
GAP-43GFP
0
1
5
20
Time in TX-100 (min)
S P S P S P S P
← p62c-yes
← Chimeric GFPs
← caveolin
Coomassie Stained PVDFs
B
S P
77
50
32
21
← mouse anti-p62c-yes
← rabbit anti-GFP
← rabbit anti-caveolin
C
S P
77
50
32
21
*
← mouse anti-p62c-yes
← rabbit anti-GFP
← rabbit anti-caveolin

Figure 3.12. Solubilization kinetics of acylated GFPs, caveolin, and p62$^{c\text{-}yes}$. COS-7 cells expressing various acylated and nonacylated GFP chimeras were separated into 4°C TX-100-soluble and resistant fractions by incubating cell monolayers with 1% TX-100 for 0-20 min. The 1% TX-100 fractions containing solubilized total cellular proteins were rapidly removed from the tissue culture dishes, and the detergent-resistant matrices solubilized with a 1X lysis buffer solution. These separated fractions were immunoblotted with rabbit anti-GFP (1:2000), anti-caveolin (1:4000), and mouse anti-Yes (1:1000) Abs. (A), immunoblots showing relative distributions of acylated GFPs, caveolin, and p62$^{c\text{-}yes}$ between TX-100-soluble (S) and -resistant (P) fractions. Markers, right, point to the position of migration of the indicated proteins. Below each immunoblot is a corresponding Coomassie-stained PVDF membrane showing the kinetic distribution of total cellular protein between TX-100-soluble and -resistant fractions. (B), immunoblot showing the specificity of the three Abs used in this analysis. YesGFP is being detected in the anti-GFP blot. (C), pairwise Ab controls demonstrating lack of crossreactivity between the three Abs. Detected bands are indicated by asterisks.

3.5.1 All Myristoylated GFPs Containing a Second Membrane Association Signal Colocalize with Cholesterol and Sphingolipids but not Caveolin

Myristoylated GFPs containing two membrane association signals (myristoylation plus palmitoylation or myristoylation plus polybasic region) localize similarly to a variety of endocytic structures including early endosomes, endosomal recycling compartment, late endosomes as well as lysosomes and the plasma membrane in COS-7 cells. Endosomes are heterogeneous and known to be enriched in cholesterol (Montesano *et al.*, 1981). They also differ widely in structure, shape, lipid composition, function, and pH values (Teter *et al.*, 1998). Consistent with our results, several variably lipidated proteins recently have been found to be associated with cholesterol-rich vesicles (Roy *et al.*, 1999). We also show that a dually palmitoylated (but not myristoylated) GFP, that we previously found associated with the Golgi apparatus and the plasma membrane, colocalized primarily with membranes enriched in cholesterol in the perinuclear Golgi including the TGN compartment, as well as the plasma membrane. Thus, we conclude that different combinations of acylation signals confer association with different membranes enriched in free cholesterol. This suggests that protein fatty acylation may provide a signal for recruitment or retention into cholesterol-rich membranes.

Cholesterol is a major component of the plasma membrane and endosomes, and is enriched along with sphingolipids in DRMs (Brown and London, 1998b; Ilangumaran and Hoessli, 1998). Cholesterol has also been shown to be required for maintenance of caveolae structure and function (Hailstones *et al.*, 1998; Orlandi and Fishman, 1998), endocytic retention/trafficking of GPI proteins (Mayor *et al.*, 1998), and clathrin coated pit budding (Subtil *et al.*, 1999). Of special interest is the fact that free cholesterol content is essential for endocytosis and maintenance of endosomal structure (Subtil *et al.*, 1999). This stringent cholesterol requirement in endosomal biogenesis can certainly explain in part the significant accumulation of acylated GFPs at the plasma membrane and the loss of localization of chimeric GFPs to peripheral endosomal vesicular structures when transfected COS-7 cells are depleted of cholesterol. The absence of colocalization of acylated GFPs in peripheral endosomal vesicles in cholesterol-depleted cells suggest an alteration in recycling of acylated GFPs between the plasma membrane and various intracellular compartments, such as the endocytic recycling compartment or TGN (Rodal *et al.*, 1999). This possibility is further strengthened by the fact that active recycling of GM_1 is apparently severely reduced in cholesterol-depleted cells, as seen by the absence of intracellular GM_1 signal in comparison to untreated cells. The cholesterol content in membranes has been shown to be essential for proper localization of fatty acylated proteins. Cholesterol depletion experiments using MβCD significantly altered the subcellular localization of Fyn, Lck, Lyn, ecNOS, and caveolin (Ilangumaran and Hoessli, 1998; Blair *et al.*, 1999; Sheets *et al.*, 1999). Thus, our results are consistent with cholesterol playing a key role in the proper localization of fatty acylated proteins. Also, combinations of myristoylation and palmitoylation or polybasic domain may represent a form of PM localization signal that are poor at PM retention, thereby ending up in endosomes. Since cholesterol is required for endosome formation, the presence of sphingolipids and cholesterol in these endosomal membranes may be incidental and perhaps some other feature of these membranes are responsible for the targeting of acylated GFPs to these structures.

The ganglioside GM_1 has been reported to be enriched fourfold in caveolae as identified by the colocalization of gold-CTX B subunit with caveolin (Parton, 1994). Consequently, GM_1 and caveolin have become common markers for the identification and purification of caveolae (Orlandi and Fishman, 1998). Despite this, the absence of colocalization of acylated GFPs (except for GAP-43GFP) with caveolin, and localization of acylated GFPs to GM_1-rich membranes suggest that other GM_1-containing membranes exist. This conclusion is supported by several reports which suggest that GM_1-containing membrane domains are believed to exist distributed randomly in coated pits or in clustered DRM rafts/glycolipid microdomains (Parton, 1994; Orlandi and Fishman, 1998; Sorice *et al.*, 1999). The fact that all acylated GFP chimeras are found in membranes (plasma membrane domains and internal membranes) enriched in GM_1 suggest that sphingolipids, in addition to cholesterol, may play a role for proper localization of acylated GFPs. From a mechanistic point of view, our localization data are consistent with biophysical partitioning of acylated GFPs into liquid-ordered (l_o) membrane domains known to be enriched in cholesterol and sphingolipids (Brown and London, 1998a; Schroeder *et al.*, 1998). Such simple biophysical partitioning of acylated GFPs to specific membranes could represent an efficient means to simplify the sorting of variably acylated proteins throughout the variety of cellular membranes.

3.5.2 All Myristoylated GFPs Containing a Second Membrane Association Signal do not Cofractionate with Caveolin-Rich Membranes or DRMs

All our myristoylated GFPs containing a second membrane association signal did not colocalize significantly with caveolin, and did not cofractionate with caveolin-rich membranes or DRMs prepared by two different isolation procedures. This suggests that combination of myristoylation and palmitoylation or myristoylation and a polybasic domain are neither sufficient to confer apparent colocalization with caveolin nor to allow cofractionation with DRMs. As such, our results imply that our acylated GFPs are found in cholesterol-, sphingolipid-enriched membranes (based on colocalization data) and are TX-100 detergent soluble (based on biochemical fractionation data). These data are also consistent with the fact that only a proportion of cholesterol (26%) and gangliosides (67%) are TX-100 insoluble in MDCK cells (Brown and Rose, 1992). Thus, substantial amounts of these cellular lipids do exist in a detergent-soluble state (Janes *et al.*, 1999). GFP itself does not cause exclusion of our GFP chimeras or other proteins from lipid rafts. Other reports have utilized GFP fusion proteins that target effectively to lipid rafts and caveolae (Volonte *et al.*, 1999; Nichols *et al.*, 2001; Pyenta *et al.*, 2001).

To further characterize the membrane fractionation properties of our acylated GFPs in comparison to known lipid raft markers, we utilized endpoint and kinetic solubilization studies investigating the fractionation of known lipid rafts/DRM markers in COS cells in the presence of a variety of detergents. Our results showed proper fractionation of Yes PTK and caveolin into lipid rafts, corroborating previous fractionation results (Melkonian *et al.*, 1995). In contrast, acylated GFPs were readily soluble in all detergent types and conditions tested. However, these latter results appear to be in direct contradiction with those of Galbiati and coworkers, who studied the fate of a short myristoylated and palmitoylated 32 amino acid $G\alpha_i$ sequence appended to GFP (Galbiati *et al.*, 1999a). With the first eleven amino acids of $G\alpha_o$ and $G\alpha_i$ being identical (our $G\alpha_o$GFP chimera contains 11 amino acids from $G\alpha_o$) the discrepancy may reside in the extra amino acids appended to GFP. Indeed, from the three-dimensional X-ray structure of several heterotrimeric $G\alpha$ subunits, it appears that amino acids 9-32 are part of an α-helix (Lambright *et al.*, 1996). Furthermore, Busconi and Denker demonstrated the importance of amino acids 11-14 of $G\alpha_o$ in membrane binding, with deletion leading to substantial reduction in membrane affinity (Busconi *et al.*, 1997; Busconi and Denker, 1997). They also suggested that the N-terminus of $G\alpha_o$ interacts with another protein in the acceptor membrane, independent of $\beta\gamma$ binding. Perhaps this α-helix also forms independently when appended to GFP and contributes specific protein-protein interactions involved in colocalization and co-fractionation with caveolin.

$p62^{c\text{-}yes}$, which was shown to colocalize with YesGFP (McCabe and Berthiaume, 1999), minimally colocalized with caveolin, but did cofractionate with caveolin-rich lipid rafts/DRMs independent of the isolation procedure used to prepare lipid rafts or DRMs (*i.e.* detergent-free and detergent-based methods). Our results confirmed those of others who showed that $p62^{c\text{-}yes}$ is found on recycling endosomes and plasma membrane, and when expressed in MDCK cells is found in the TX-100 insoluble fraction (Arreaza *et al.*, 1994; Melkonian *et al.*, 1995; Luton *et al.*, 1999). A minimal explanation for these results is that $p62^{c\text{-}yes}$ and YesGFP are both localized in cholesterol-, sphingolipid-enriched membranes of varying detergent solubility, and that $p62^{c\text{-}yes}$ is localized in DRMs/lipid rafts that are different than those containing caveolin. These results are consistent with the prior demonstration of the existence of different types of DRMs/lipid rafts (Iwabuchi *et al.*, 1998; Orlandi and Fishman, 1998; Waugh *et al.*, 1998; Hooper, 1999). This observation is also consistent with the requirement of protein-protein interactions between $p62^{c\text{-}yes}$ and a given acceptor protein as a prerequisite to acquire detergent-resistance/lipid raft localization.

In direct contrast to the results obtained with $p62^{c\text{-}yes}$, dually palmitoylated GAP-43GFP chimera colocalized with caveolin (in the Golgi area but not at the plasma membrane) but did not cofractionate with caveolin-rich DRMs. This may reflect common initial targeting and acylation of caveolin and GAP-43 at the level of the Golgi. Furthermore, caveolin and GAP-43 are known to have Golgi targeting domains and palmitoylation-deficient mutants of caveolin and GAP-43 showed negligible association with the Golgi complex. Interestingly, targeting to the plasma membrane is not required for palmitoylation of caveolin and GAP-43 (Liu *et al.*, 1994; Galbiati *et al.*, 1999b; Luetterforst *et al.*, 1999; McLaughlin and Denny, 1999). Our findings support the requirement for protein-protein interactions as a prerequisite for localization to lipid rafts/DRMs, since full-length GAP-43 (but not our shortened GAP-43GFP chimera) was found in DRMs in COS-7 cells and neurons (Arni *et al.*, 1998). Also in this report, Brown and coworkers found DRM association with a 20 amino acid sequence of GAP-43 appended to β-galactosidase (NM_{20}-β-gal). As native β-gal is tetrameric, oligomerization of NM_{20}-β-gal would combine eight palmitate chains for membrane association, thereby indirectly promoting DRM association through multiple palmitoylation. Also, in support of the protein-protein interaction requirement for caveolae localization are the recent results of Prabhakar *et al.* (Prabhakar *et al.*, 2000). They showed that a nonacylated chimeric ecNOS (normally myristoylated and dually palmitoylated) with a transmembrane domain directs ecNOS to caveolae, suggesting that acylation is not required for selective targeting of ecNOS to caveolae, but rather sequences within the protein specify the caveolar association.

3.5.3 Requirements for Fatty Acylation-Dependent Subcellular Localization to Cholesterol-, Sphingolipid-Rich Membranes and Lipid Rafts/DRMs

Figure 3.13 depicts a model integrating the facts that 1) all of our fatty acylated chimeric GFPs were detergent-soluble and found in cholesterol, sphingolipid-enriched membranes (based on our indirect fluorescence microscopic co-localization and TX-100 solubilization/fractionation results); 2) all of our acylated GFPs (except dually palmitoylated GAP-43) did not colocalize with caveolin (based on our indirect immunofluorescence results); 3) none of our acylated GFPs were found in lipid rafts prepared using standard detergent or detergent-free methods; and 4) in addition to proper fatty acylation, protein-protein interactions (*e.g.* perhaps via SH2 or SH3 domains) are required for $p62^{c\text{-}yes}$ to cofractionate with noncaveolar DRMs (based on the fact that $p62^{c\text{-}yes}$ did not colocalize with caveolin in our indirect immunofluorescence experiments but did cofractionate in caveolin enriched DRM/lipid rafts). Our model is similar to those of Ilangumaran and Hoessli and Madore *et al.*, who also depict lipidated proteins in membrane domains of varying levels of liquid order state and detergent-solubility (Ilangumaran and Hoessli, 1998; Madore *et al.*, 1999). It should be noted that this model is only one interpretation of the available data. Other models to which the data fit are possible. One alternate model that explains the results may be that the detergent resistant and detergent soluble lipid "rafts" may exist as wholly separate entities in the membrane. These adjacent areas may be contiguous in the membrane, and smaller than the optical resolution of the confocal microscope. As such, YesGFP and YesPTK may be in different rafts. Importantly, however, the biochemical and morphological data presented in this thesis are consistent wth acylated GFPs not being in rafts. As such, it may be concluded based on the data that acylated GFPs are not in membrane subdomains at all, but rather localize to the bulk endosomal, Golgi and PM membranes. Future developments in techniques that can assess membrane subdomains in their native state will need to be developed to conclusively analyze lipid rafts and the presence/absence of particular proteins.

In **Figure 3.13**, acylated GFPs, rather than associating with DRM core areas, may lie juxtaposed in an annulus rich in cholesterol and sphingolipid that is TX-100 soluble. As lipid rafts have been defined operationally by detergent insolubility or co-association with caveolin and other lipid raft markers, our GFP chimeras are not in rafts by definition. This localization reconciles the fact that YesGFP and $p62^{c\text{-}yes}$ colocalize by immunofluorescence analysis, yet only $p62^{c\text{-}yes}$ cofractionates with caveolin-rich DRMs/lipid rafts. Based on our previous observations demonstrating that combined addition of myristoylation and palmitoylation (*e.g.* YesGFP) or myristoylation plus polybasic domain (*e.g.* Src_{16}GFP) onto GFP conferred similar localization to plasma

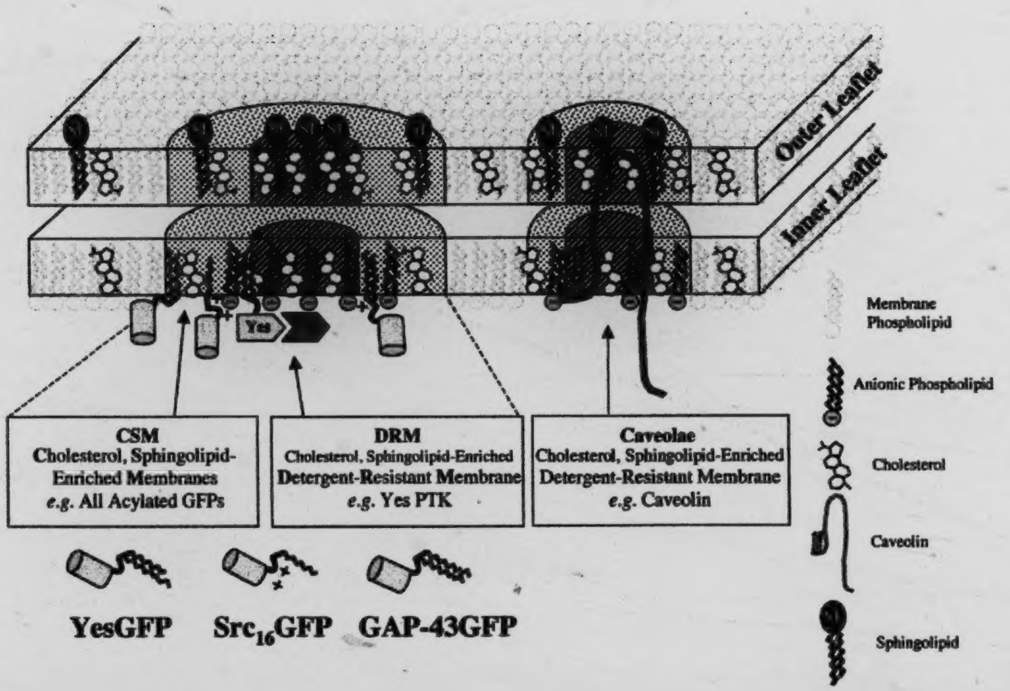
Outer Leaflet
Inner Leaflet
Yes
CSM
Cholesterol, Sphingolipid-Enriched Membranes
e.g. All Acylated GFPs
DRM
Cholesterol, Sphingolipid-Enriched Detergent-Resistant Membrane
e.g. Yes PTK
Caveolae
Cholesterol, Sphingolipid-Enriched Detergent-Resistant Membrane
e.g. Caveolin
YesGFP
Src16GFP
GAP-43GFP
Membrane Phospholipid
Anionic Phospholipid
Cholesterol
Caveolin
Sphingolipid

Figure 3.13. Model of fatty acylation-dependent membrane localization. The model depicts the localization of acylated GFPs with cholesterol and sphingolipid-enriched membrane microdomains (CSMs) (dotted area). All three types of membrane association signal combinations (myristoylation plus palmitoylation: YesGFP; myristoylation plus polybasic region: Src_{16}GFP; and dual palmitoylation plus polybasic region: GAP-43GFP) showed similar localization at the plasma membrane. Acylated, full-length Yes PTK is shown in the cholesterol and sphingolipid-enriched detergent resistant membrane fraction (DRM) (hatched area). Yes PTK is depicted in a DRM separate from DRMs containing caveolin, right (since Yes PTK and caveolin do not colocalize). Protein-protein interactions (represented by association with the unidentified orange protein) are believed to mediate Yes PTK association with the DRM core. For simplicity, lipid rafts present in endosomal or Golgi structures are not illustrated. Models of various lipid raft membrane constituents are shown in the legend at right.

membrane and endosomal membranes, we included negatively charged phospholipids on the inner leaflet of the plasma membrane (*i.e.* the outer leaflet of endosomes) in our model. The presence of negatively charged phospholipids in the inner leaflet of the plasma membrane is well-documented (Devaux, 1991; Zwaal and Schroit, 1997). Furthermore, it is also consistent with the fact that a significant portion of these negatively charged phospholipids are known to contain two saturated acyl chains (Holub, 1980; Fridriksson *et al.*, 1999), and that this phospholipid population has been preferentially found in cholesterol-, sphingolipid-rich lipid rafts or DRM domains (Harder and Simons, 1997; Fridriksson *et al.*, 1999). In addition, endosomal membranes, which can support a liquid-ordered membrane phase (Brown and London, 1998b), are known to be the most net negatively charged of all cellular membranes (Cavenagh *et al.*, 1996).

Also noted in our kinetic solubilization experiments was the difference in solubilization rates between $p62^{c\text{-}yes}$ and caveolin. These differences may reflect differences intrinsic to protein structures, but could also be interpreted as $p62^{c\text{-}yes}$ and caveolin residing in different DRM membrane environments. This possibility would further confirm the absence of colocalization between the two proteins in our indirect immunofluorescence experiments. Thus, we depicted at least two types of DRMs or lipid rafts in our model. In various reports, several acylated signaling proteins were shown to be enriched in DRM fractions, but in those studies whether these signaling proteins actually colocalized with caveolin or not was not assessed (Arreaza *et al.*, 1994; van't Hof and Resh, 1997; Arni *et al.*, 1998; Galbiati *et al.*, 1999a; Melkonian *et al.*, 1999).

According to our data and those of others, we believe that N-terminal acylation acts primarily as a sensor/retention signal for cholesterol-, sphingolipid-enriched membranes. Our results are in agreement with the kinetic bilayer trapping mechanism of Shahinian and Silvius, in which myristoylated GFPs would sample various cellular membranes rich in saturated lipids, sphingolipids and cholesterol (Shahinian and Silvius, 1995). Then, the presence of a polybasic domain adjacent to the myristate, through electrostatic attractions, would concentrate that myristoylated protein in membrane domains rich in sphingolipids, cholesterol, and saturated anionic phospholipids of endosomes and plasma membrane. Alternatively, myristoylated proteins could sample membranes until reaching a proper membrane environment containing a putative PAT enzyme. A productive interaction between the two could lead to the addition of a second saturated fatty acid on the myristoylated protein which would further enhance its partitioning into liquid ordered cholesterol-, sphingolipid-enriched membrane domains. Such partitioning would thus restrict the number of membranes containing dually acylated proteins, facilitate their intracellular sorting and stimulate potential protein-protein interactions with other membrane bound proteins. Upon establishment of favorable protein-protein interactions, the complex could then move from the periphery (annulus) of cholesterol-, sphingolipid-enriched membrane domains to a core TX-100 insoluble membrane domain. We make a critical addition to the kinetic bilayer trapping mechanism by including protein-protein interaction modules as part of the membrane "trapping" mechanism. The importance of protein-protein interactions in localization of proteins to DRMs/lipid rafts is also supported by work on G protein subunits, PSD-95, ecNOS, and linker for activation of T cells (LAT) (Busconi and Denker, 1997; Craven *et al.*, 1999; Fishburn *et al.*, 2000; Harder and Kuhn, 2000; Prabhakar *et al.*, 2000). In these articles, the final destination or localization of a given fatty acylated protein was shown to be further altered or modified by protein modules (caveolin binding domain or CBD, SH2, SH3, PDZ, βγ subunit binding region, *etc.*) to combinatorially determine the ultimate steady state destination of that protein.

CHAPTER 4

GENERAL DISCUSSION

4.1 EXPERIMENTAL SUPPORT OF THE MEMBRANE BINDING MODELS INVOLVING N-TERMINALLY ACYLATED PROTEINS

The data presented in this thesis describe the differential behaviour of variably acylated N-terminal GFP chimeras in localizing to membrane and submembrane compartments, as assessed through gain-of-function analyses using radiolabeling, subcellular fractionation, live cell confocal microscopy, biochemical and immunocytochemical techniques. The scope of this study is unique in that it collectively compares many wild-type and mutant acylated protein sequences appended to a fluorescent reporter in living and fixed COS-7 cells. The chimeras demonstrated acylation-dependent differences in subcellular localization, fractionation behaviour, and in membrane association. This study represents a modification from the current widely held belief that covalently attached lipids to proteins alone mediates special biophysical partitioning into uniquely composed detergent-resistant "lipid raft" areas of the plasma membrane, endosomes, or Golgi apparatus. Much of the literature has based the involvement of acylation in membrane association on the loss of membrane targeting when the amino acid residues subject to acylation were mutated. The results presented here confirm and augment the emerging model of the role of N-terminal acylation in protein localization. By focusing in on the role of the lipid modified sequences outside their normal context (*i.e.*, in the full-length protein, where numerous other protein and membrane interacting factors may be at play), a view of what N-terminal acylation does free of protein-protein interactions can be achieved. We clearly show that N-terminal acylation is necessary for localizing proteins to endosomes, Golgi, and plasma membranes, and to exclude the proteins from the nucleus, especially evident in living cells. However, N-terminal acylation is not sufficient to mediate strong association with detergent-insoluble membrane domains in our study system. Significant evidence has accumulated in the literature that other factors mediate association with either caveolae or DRMs, especially protein-protein interactions and the formation of supramolecular signaling clusters, often with significant protein crosslinking and involvement of the cytoskeleton (Fanning and Anderson, 1999; Prabhakar *et al.*, 2000). Evidence has been presented that this alone may lead to insolubility in various detergents for a number of membrane associated proteins (Hare and Holocher, 1994).

As mentioned in Chapter 1, various models for lipidation-dependent membrane association of proteins have been proposed. Our experimental results lend support for these models. Myristoylated proteins interact weakly and transiently with intracellular membranes. The addition of a second signal such as palmitoylation traps the protein in the membrane where palmitoylation occurred, consistent with the kinetic bilayer trapping model and two signal hypothesis for membrane binding. These appear to be the operative mechanisms for myristoylated and palmitoylated GFP chimeras studied in this project (Fyn, Yes, $G\alpha_o$, Lck). Fyn(C3,6S)GFP and Yes(C3S)GFP were critical constructs to further support the model as evidenced by their ER association, minimal PM association, and significant presence in the soluble fraction in subcellular fractionation experiments. However, an unexpected association with endosomes was noted (see **Section 4.2**), suggesting that myristoylation provides a significant enough signal to bind to endosomes, or that in addition to myristoylation, palmitoylation could be required for transport to the PM. For constructs bearing a myristoylation signal and a polybasic region such as Src_{16}GFP, our results support the surface potential sensor model where myristate coupled with the electrostatic interaction of the basic residues with anionic phospholipids leads to association with target membranes. As noted in **Figure 3.8**, at t = 0 min, a significant portion of the Src_{16}GFP construct was associated with the soluble fraction even when transiently exposed to a detergent (TX-100) solution (<1 sec), in contrast to the dually acylated YesGFP and GAP-43GFP chimeras. This strongly suggests that Src_{16}GFP binding may be less stable or reversible when assessed biochemically, yet appear morphologically the same by confocal microscopy. These results are similar to studies looking at membrane association of farnesylated plus polybasic domain K-Ras4B (Leventis and Silvius, 1998; Roy *et al.*, 2000). Binding of a model fluorescent peptide and C-terminal sequence GFP chimera was shown to be high but rapidly reversible ($t_{1/2}$ of seconds) to membranes containing a negatively charged surface. These molecules were abstractable by negatively charged lipid vesicles, showed no preference for specific anionic lipids, and were tolerant of mutations to the targeting motif without loss of function if the overall strongly polybasic/amphiphilic character was maintained (Roy *et al.*, 2000).

4.2 DIFFERENCES BETWEEN BIOPHYSICAL STUDIES *IN VITRO* AND EXPRESSION ANALYSIS *IN VIVO* OF ACYLATED PEPTIDES/PROTEINS

In comparison to previous studies, myristoylated only mutants Yes(C3S)GFP and Fyn(C3,6S)GFP displayed significant association with endosomal membranes, as detected by colocalization with DiI-LDL and by subcellular fractionation. Our myristoylated chimeric GFPs appeared to associate more avidly with membranes than corresponding lipidated peptides (Shahinian and Silvius, 1995). This may be due to contributions of the GFP molecule, electrostatic interactions, viscosity of the cytoplasm, or to the fact that the composition of cellular membranes is far different and more complex than *in vitro* model membranes. Our observation is also supported in the literature by other reports (van't Hof and Resh, 1997; Wolven *et al.*, 1997). Fusion of a myristoylated, nonpalmitoylated Fyn sequence to β-galactosidase, a cytosolic protein that can form tetramers (Fyn_{16}(C3,6S) β-gal), led to membrane association of 32%, whereas a Fyn_{25}(C3,6S)GST fusion was 78% membrane-bound. Clearly, additional factors must be at play to explain the discrepancy in the two results, which would be expected to be equivalent.

Silvius and coworkers have also provided evidence supporting the higher than expected association of myristoylated only chimeric GFPs. They reported that phospholipid species bearing monounsaturated or shorter-chain saturated species (*e.g.* myristate) partition into liquid-ordered membrane phases with significant yet modest affinities, and that this partitioning is underestimated by using the criterion of low-temperature detergent insolubility (Wang *et al.*, 2000). These authors also report that oligomerization of lipid-modified species may enhance their relative affinities for liquid-ordered phase domains many fold. It is also possible that via protein-protein interactions mediated by the myristate, these chimeras cluster/aggregate/self associate and generate enough hydrophobic character to partition into endosomal membranes (Roy *et al.*, 1997).

4.3 INFLUENCE OF REPORTER PROTEIN SELECTION ON RESULTS AND INCLUSION OF A LINKER REGION TO ALLOW PROPER ACYLATION

Work described in Chapter 2 outlined the advantages of using the xenobiotic fluorescent protein GFP as a reporter protein in studying acylation-mediated protein localization. To date, there are no known reports of this protein binding to any structures inside eukaryotic cells used as expression systems. However, the cytosolic surface of the 11 β-strand barrel quaternary structure could possibly influence the ability of the protein to interact with membranes (Ormo *et al.*, 1996; Yang *et al.*, 1996), cause steric effects, and allow some electrostatic interactions to impact on the membrane partitioning of lipid-modified GFP proteins (Pool and Thompson, 1998). Reconciling this is the fact that with the addition of a seven amino acid flexible neutral hydrophilic linker (TKLTEER) from Fyn between the acylated sequences and GFP sequence, all chimeric proteins would be subject to identical effects on membrane binding caused by the protein and linker. This linker appears to be critical, as the acylated residues of many lipidated proteins are found within flexible regions of the protein (random coil) (Buser *et al.*, 1994; Yeagle *et al.*, 1996; Murray *et al.*, 1998; Sutton *et al.*, 1998; Victor and Cafiso, 1998; Druey *et al.*, 1999; Langen *et al.*, 1999). Absence of a flexible linker between the acylation sequence and GFP can have deleterious consequences such as inefficient acylation, as in the case of AKAP18, $G\alpha_i$, $G\alpha_o$, and ecNOS (Busconi and Denker, 1997; Liu *et al.*, 1997a; Fraser *et al.*, 1998; Galbiati *et al.*, 1999). In addition to creating an extension potentially preventing steric hindrance to NMT and PAT, the choice of a single linker sequence from Fyn PTK (residues 12-18) was made to facilitate the creation of various chimeric fusion proteins by using a common oligonucleotide. An alternative strategy would be to use the homogeneously neutral charged linker such as polyGlyAla. This would remove the possibility of hydrophilic/basic residues in the TKLTEER sequence contributing to membrane association. As such, the polar TKLTEER sequence was found in all the chimeric GFPs. Serendipitously, a recent paper outlining the proteomics of NMT substrates and the refinement of the consensus sequence identified three motif regions in myristoylated proteins. Positions 1-6 fit the binding pocket, positions 7-10 interacted with the surface of NMT at the mouth of the catalytic cavity, and positions 11-17 comprised a hydrophilic linker (Maurer-Stroh *et al.*, 2002).

It was reported during the course of this project that the N-terminus of Fyn amino acid residues 1-19 interacts with Tctex-1, a light chain of the dynein cytoskeletal motor complex through yeast two hybrid analysis (Campbell *et al.*, 1998). This dynein light chain has been found to localize predominately to the Golgi apparatus (Tai *et al.*, 1998).

Further truncation to Fyn 1-10 abrogates Tctex-1 interaction. Fortunately, all chimeric constructs other than FynGFP (which is Fyn$_{18}$GFP) contain only amino acids 12-18 of Fyn, which would not be sufficient for Tctex-1 association. As there was no difference in the subcellular localization between related NRTK-GFP fusions FynGFP (possible Tctex-1 binding) and YesGFP (no Tctex-1 binding), it seems unlikely that this potential interaction is creating an artifact of localization in our system. This dynein complex including Tctex-1 is responsible for microtubule (-)-end-directed organelle movement, endosomal transport, centrosomal localization of the Golgi complex, chromosome segregation, mitotic spindle alignment and nuclear distribution (Tai *et al.*, 1998). It is striking that the N-terminus of Fyn, dually acylated, binds to Tctex-1, and the subsequent discovery of a Tctex-1 binding site in the C-terminal dipalmitoylated tail of rhodopsin (Tai *et al.*, 1999). This is one of the first similarities between peripheral and integral membrane palmitoylated proteins beyond GPCR-G protein coupling (*e.g.* β_2-adrenergic receptor and Gα_s), suggesting that acylation, vesicular/endosomal trafficking and the cytoskeleton may be linked in ways still poorly understood.

Work described in Chapter 2 demonstrated initial attempts to characterize the role of various N-terminal acylation motifs in subcellular localization. Significantly, we showed that sequences that conferred different combinations of acylation and polybasic domains mediated specific subcellular localization in a eukaryotic expression system. We showed that protein motifs isolated from different signaling proteins, containing different numbers of fatty acids attached, different positions of attachment, and different fatty acids attached to GFP, were localized to different sites in COS-7 cells. Myristoylated only proteins demonstrated widespread endomembrane association, nuclear exclusion, with minimal association with the plasma membrane. Myristoylated plus increasing amounts of net electrostatic charge via basic amino acid residues, exemplified by the construct Src$_{14}$GFP (net charge of appended sequence: +2), demonstrated increasing amounts of plasma membrane and focal perinuclear vesicular association. In Src$_{16}$GFP (net charge of appended sequence: +5), the morphology of association did not differ from that of myristoylated and palmitoylated GFP chimeras. Myristoylated and palmitoylated proteins all localized to endosomes and the plasma membrane. A dipalmitoylated GAP-

43GFP displayed plasma membrane and weak perinuclear fluorescence in live cells, identified as Golgi association in fixed cells.

Our findings suggest that biophysical properties of acyl chains and polybasic domains of N-terminally acylated sequences are mediating the association and enrichment with certain cellular membranes. Such results argue against a requirement for a specific amino acid sequence, and are not consistent with a proteinaceous receptor of acylated proteins mediating membrane association via protein-protein or protein-lipid interactions.

4.4 FATTY ACYLATION – A MODIFICATION KEEPING PROTEINS OUT OF THE NUCLEUS

In our studies, no acylated protein chimeras, whether myristoylated and/or palmitoylated, were found in the nucleus. This was especially evident under live cell conditions, where the artifacts of fixation and processing were avoided.

Evidence of acylated intranuclear proteins is scant. The only three documented cases in the literature involve viral proteins, HIV-1 Gag and polyoma virus/SV40 VP2 proteins, and the catalytic subunit of PKA (Streuli and Griffin, 1987; Gallay *et al.*, 1995; Pepperkok *et al.*, 2000). In these cases, structural elements may bury the myristate inside the protein, not exposing it to cytosolic factors and allowing transit through the nuclear pore. Isoforms of acylated proteins exist that lack a myristoylation motif and are localized to the nucleus, such as the NRTKs Arg and Abl, or lack a palmitoylation motif, as seen in SNAP-25A/SNAP-25B and Rlk/Txk (Bark and Wilson, 1994; Debnath *et al.*, 1999). For example, with Rlk/Txk, the shorter protein species lacks a cysteine string motif and is located in the nucleus; in contrast, the larger form is membrane-bound and palmitoylated. Thus, the mutation of its cysteine string motif both abolishes palmitoylation and allows the protein to migrate to the nucleus. The cysteine string, therefore, is a critical determinant of both fatty acid modification and protein localization for the larger isoform of Rlk. For the case of IL-1α, internal lysine myristoylation of the N-terminal propiece disrupts a nuclear localization sequence (NLS) motif that would normally target protein to the nucleus, where, if overexpressed, it acts as a transforming nuclear oncoprotein causing transactivation of specific immune response genes (Stevenson *et al.*, 1997).

Overall, a role for fatty acylation in nuclear exclusion is inferred from our experimental results, as well as reports in the literature. Many fatty acylated proteins are signal-transducing proteins such as kinases and G proteins, with potential to cause serious cellular alterations if activated in the wrong location. It may be inferred that acylated isoforms of proteins prevent nuclear localization (David-Pfeuty *et al.*, 1993).

4.5 SUPPORT OF THE LIPID RAFT MODEL AS THE MECHANISM FOR MEMBRANE LOCALIZATION OF N-TERMINALLY ACYLATED PROTEINS

At the microscopic level, the plasma membrane localization of myristoylated plus palmitoylated, myristoylated plus polybasic domain, and dipalmitoylated GFP chimeras was similar. Evidence has continued to accumulate supporting the concept that certain membranes are enriched in particular molecular species, such as cholesterol, sphingolipids, and anionic phospholipids. Polybasic domain proteins and peptides have been hypothesized to create their own microdomains by clustering anionic phospholipids (Denisov *et al.*, 1998; Arbuzova *et al.*, 2002; Rauch *et al.*, 2002). Such common localization may be simply due to the fact that acylated proteins prefer cholesterol, sphingolipid-rich membranes, and acylated plus polybasic domain proteins prefer membranes with anionic phospholipids, and these may be the same membranes.

We demonstrated in Chapter 3 that our acylated constructs were found in membranes enriched in free cholesterol and sphingolipids, with myristoylated plus polybasic/palmitoylation GFP chimeras colocalizing in endosomes and plasma membrane, and dipalmitoylated GAP-43GFP colocalizing in the Golgi and PM. This association with cholesterol and sphingolipids was shown to be critical, as acute depletion of cholesterol with MβCD led to alterations in cell morphology, reduction in endocytosis, affecting the location of the acylated protein, free cholesterol and sphingolipid GM_1. This illustrates the connection between acylated proteins, cholesterol, and sphingolipids, and lends support to the lipid raft theory, which predicts that these cellular constituents would be associated due to a biophysical propensity to cluster into liquid-ordered domains. In addition to the PM lipid raft/DRM association of acylated proteins, a recent report demonstrated in living cells that a significant proportion of cellular cholesterol was found in the endosomal recycling compartment (ERC) (Hao *et* *al.*, 2002), a compartment enriched in GPI-anchored proteins which possessed cholesterol-dependent retention as compared to rapid PM-recycling endocytic receptors such as the transferrin receptor (Mayor *et al.*, 1998). As well, this compartment was a filipin-positive, transferrin-positive compartment to which acylated GFP chimeras in this study localized (McCabe and Berthiaume, 2001). Despite the lack of stable DRM association, our results support the concept that association with lipid raft components in a detergent-soluble fashion may be the initiating event of localization to DRMs/lipid rafts, especially if such a role was to facilitate the manifestation of other stronger (*i.e.* protein-protein) interactions.

4.6 CAVEOLIN/CAVEOLAE AND ACYLATED PROTEINS: ASSOCIATION COINCIDENCE?

From our studies on caveolin localization within COS-7 cells, it can be safely concluded that the distributions of this protein do not match well with any acylated construct used in this study, although there was partial colocalization with GAP-43GFP in the Golgi region. This is explained by the fact that both proteins are believed to have a Golgi targeting signal in their protein sequence (Aarts *et al.*, 1995; Luetterforst *et al.*, 1999). Thus, caveolae, as specialized DRM regions, do not appear to obviously mediate N-terminal acylated protein localization in this study system. Caveolae and caveolin have been difficult to study, and discrepancies in the literature exist about their role and function (Kurzchalia and Parton, 1996; Pfeffer, 2001; Thomsen *et al.*, 2002). The presence of N-terminally acylated proteins within caveolae may have more to do with the fact that the majority of these signaling proteins contain a hydrophobic caveolin-binding domain. Thus, the association with caveolae may be due primarily to protein-protein interactions, and not interactions of lipid-modified protein acyl chains with caveolar lipids (Li *et al.*, 1996; Okamoto *et al.*, 1998). This has been conclusively shown for myristoylated and dually palmitoylated ecNOS (Feron *et al.*, 1998; Prabhakar *et al.*, 2000).

4.7 N-TERMINAL ACYLATION IS NOT SUFFICIENT FOR DRM ASSOCIATION

Prior to conducting experiments assessing the lipid raft association of our GFP chimeras, it was hypothesized that our constructs would associate favorably with these membranes, based on the previous literature in the field. To our surprise, using sucrose gradient fractionation with detergent-present and detergent-free methods, our acylated GFP chimeras did not cofractionate with known caveolae/DRM proteins caveolin and $p62^{c\text{-}yes}$. This is contrary to some reports in the literature (Galbiati *et al.*, 1999; Janes *et al.*, 1999; Wang *et al.*, 2001a). Reasons for the discrepancy include the study of different acylated proteins, cell types, and fractionation methods. Such items could greatly influence the results of such studies. Of note, the reports by Janes *et al.* and Wang *et al.* used the same method initially used by Magee and coworkers (Zlatkine *et al.*, 1997), and this method was not used in this study. Also contributory is the length of the acylation sequence appended to GFP; Galbiati *et al.* appended 32 amino acids of $G\alpha_{i1}$, which included a membrane-interacting α-helix that has been shown to be critical for membrane association (Busconi *et al.*, 1997; Busconi and Denker, 1997). The methods used by Galbiati *et al.* were the same as those used in this report; however, their construct lengths were longer and encompassed known membrane associating areas. Therefore, the findings of our report, using minimal sequence acylated GFP chimeras, have not been contradicted in the literature to date by direct comparison.

4.8 RAPID SOLUBILIZATION OF ACYLATED GFP CHIMERAS MAY EXPLAIN THE LACK OF DRM ASSOCIATION

The solubilization of acylated GFP chimeras, as opposed to caveolin and $p62^{c\text{-}yes}$, was interesting. Using different concentrations of TX-100, and the selective lipid raft protein solubilizer, octyl glucoside (OG), the behaviour of the DRM fractions was consistent with that reported for DRMs in the literature (Melkonian *et al.*, 1995). More caveolin distributed with the DRM under lower detergent concentrations, a phenomenon evidenced by Arni *et al.* for other lipid-modified proteins such as GAP-43 expressed in COS cells (Arni *et al.*, 1998). OG effectively solubilized the majority of caveolin, meeting one of the key diagnostic criteria for DRM association (Arni *et al.*, 1998). Thus, we are confident that a true DRM fraction was isolated with our protocol. In spite of this, acylated GFPs were not found in DRMs.

A novel approach to assess the lack of DRM association was undertaken to further explore reasons for the lack of DRM association. By looking at brief exposures of cells *in situ* to TX-100, a kinetic pattern of solubilization was identified for acylated GFPs as well as for known DRM-associated proteins caveolin and p62$^{c\text{-}yes}$. In contrast to caveolin and p62$^{c\text{-}yes}$, acylated GFPs were DRM-associated if one kept the contact of the cell membrane with TX-100 brief (*i.e.* < 5 min). Beyond this time point, all acylated GFPs were solubilized by detergent. It is interesting to note that the raft association studies by Resh and coworkers precisely used such brief TX-100 exposure times in their DRM isolation protocol, which was used with minor modifications in this study (van't Hof and Resh, 1997; Wolven *et al.*, 1997).

4.9 FAILURE OF SECRETORY PATHWAY INHIBITORS TO INHIBIT PLASMA MEMBRANE ASSOCIATION SUGGESTS NOVEL TRAFFICKING MECHANISMS OF ACYLATED GFPS

No effect could be demonstrated when COS-7 cells were incubated for varying lengths of time (1, 4 and 15 h) with Brefeldin A (BFA, 10 μg/mL), or subjected to 15°C cold temperature block for 1-4 h (16 h post-transfection). These treatments are known to disrupt vesicular trafficking (Matlin and Simons, 1983; Lippincott-Schwartz *et al.*, 1990). Even in cells treated immediately with BFA after transfection, endosomal/Golgi and PM association of acylated GFP chimeras could be detected (results of these experiments are identical to distributions shown in **Figure 2.3**). While these studies were neither exhaustive nor quantitative, they suggested that N-terminal acylated proteins do not require classic vesicular trafficking to reach the PM. These observations further support the biophysical models discussed earlier, as the cell would not require energy-dependent vesicular movement to localize these proteins to their final destinations. Membrane association and retention of fluorescent lipopeptides could be seen under reduced temperature conditions at which vesicular transport would be inhibited (Schroeder *et al.*, 1996). Our work is supported by Resh and coworkers, who also saw no effect of BFA and cold temperature on the membrane association of Fyn (van't Hof and Resh, 1997).

However, our GAP-43GFP work contrasts that of Gonzalo and Linder, who demonstrated an effect of BFA and other inhibitors on newly synthesized but not mature GAP-43 (Gonzalo and Linder, 1998). It may be that even under the experimental conditions used (10 μg/ml BFA for 15 h post-transfection), a significant amount of time passed during which a stable population of GAP-43GFP reached the membrane and remained associated. As we only assessed the visual component of BFA treatment and not biochemical data, it is possible that a subtle effect was not detected under our assay conditions.

4.10 XZ PLANE ANALYSIS SUGGESTS ROLE OF N-TERMINAL ACYLATION IN CELL-CELL OR CELL-MATRIX SIGNALING

As seen in **Appendix Figure 6.4**, very interesting distributions of acylated GFPs were noted in the XZ plane (looking at the cell from the side) (our unpublished observations). All myristoylated and palmitoylated constructs showed significant enrichment along the PM adjacent to the substratum and also laterally, to give an overall "pseudobasolateral" apparent localization. Similar results were found for Src_{16}GFP, and GAP-43GFP. In contrast, myristoylated only constructs (Yes(C3S)GFP, Fyn(C3,6S)GFP) did not show such pseudobasolateral enrichment or membrane association. Nonacylated GFPs and GFP itself also did not show such enrichment. Also, cells in contact with each other appeared to be enriched in acylated GFPs at the junction (**Appendix Figure 6.5**), a finding supported by work on WT NRTK proteins at cell-cell junctions (Tsukita *et al.*, 1991). Although not polarized, COS-7 cells nonetheless possess the means to spatially regulate the appearance of acylated molecules at sites of great signaling importance: cell-cell junctions and the substratum attachment site. A link between these data and the cytoskeleton can possibly be made, as many full length acylated proteins are associated with microtubules (EF-hand Ca^{2+}-binding proteins, PSD-95, Fyn) (Ley *et al.*, 1994; Timm *et al.*, 1999; El-Husseini *et al.*, 2000), actin (MARCKS) (Hartwig *et al.*, 1992), and intermediate filaments (Yes, Src) (Ciesielski-Treska *et al.*, 1995) in these regions. It appears that these acylated chimeras also could traffic on microtubules, as endocytic vesicular structures were arranged in an organized microtubule-associated fashion when cells were probed for both acylated GFPs and α-tubulin, a microtubule component **(Appendix Figure 6.6)**.

4.11 UNEXPLAINED CONTROVERSIES REGARDING THE LIPID RAFT/DRM MODEL

According to the lipid raft theory, saturated acyl chains of sufficient length and number are required to mediate a biophysical association with liquid-ordered membrane domains. Bulky hydrophobic groups such as prenyl moieties are hypothesized to be excluded from the ordered packing of lipid chains found in lipid rafts (Brown and London, 1998b). Why then do prenylated proteins associate with DRMs or caveolae? A minimal C-terminal tetrapeptide sequence CAAX box-EGFP chimera is sufficient to localize a reporter protein to caveolae (Michaely *et al.*, 1999), and prenylated Ras isoforms and G protein βγ subunits were found significantly enriched in TX-100 insoluble DRMs in T cells (Janes *et al.*, 2000; Oh and Schnitzer, 2001). Why do these findings contradict other reports (Melkonian *et al.*, 1999; Wang *et al.*, 2000), who show that prenylated peptides/proteins have a very low affinity for detergent-resistant lipid rafts? Proteins such as Ras (Moffett *et al.*, 2000), containing a l_o-seeking lipid (palmitate) adjacent to a l_d-seeking lipid (farnesyl) within the same protein, or G protein heterotrimers (αβγ), where l_o-seeking acylation of Gα is juxtapositioned with l_d-seeking prenylation found in Gγ (Wall *et al.*, 1995; Lambright *et al.*, 1996), may constitute a unique situation in lipid raft localization.

Two models could explain the lack of DRM association of these proteins. First, if prenylation was the dominant signal, then these proteins would be in the l_d phase. Alternatively, these proteins could position themselves to the edge of l_o domains/rafts, allowing insertion of the two lipids into their respective domains. There is a certain amount of elegance to this model, as it allows for dynamic palmitoylation to mediate the transfer from the edge of DRMs into the bulk membrane. This has been suggested experimentally by Hancock and coworkers, when comparing localization of H- and K-Ras4B (Prior *et al.*, 2001), where activation of H-Ras by GTP binding translocated the protein from lipid raft to bulk-disordered PM, where it activates Raf. H-Ras and K-Ras4B, because of their different membrane association signals (H-Ras: farnesyl and palmitate; K-Ras4B: farnesyl plus polybasic domain), localize to different microregions of the plasma membrane: H-Ras to cholesterol-rich caveolin-rich microdomains, and K-Ras4B to cholesterol-poor membrane areas. Paradoxically RasGTP is the form that is supposed to recruit Raf to caveolae (Liu *et al.*, 1997b).

Also puzzling is the fact that RasGRP2, a Ras exchange factor localized to the plasma membrane via N-terminal myristoylation and palmitoylation, is an activator of K-Ras and N-Ras, but not H-Ras (Clyde-Smith *et al.*, 2000). One would have speculated that the two dually lipidated proteins (H-Ras and RasGRP2) would have interacted and presumably would colocalize in cholesterol-rich l_o phase microdomains. It may be that the RasGRP2 simply has different substrate specificities. Vac8p, the vacuole-associated protein in yeast, is N-terminally myristoylated and palmitoylated (Wang *et al.*, 2001b). It has been determined that the yeast vacuole is a sterol- (ergosterol in yeast) and SL-poor membrane, and is excluded from raft microdomains, which have been shown to exist in yeast (Bagnat *et al.*, 2000). If acylation is critical for lipid raft association, then why target this protein to a nonraft membrane?

Brown and coworkers report that transmembrane proteins, due to their lipid disorganizing TMD segments, would be disruptive in a DRM environment and would thus be excluded from lipid rafts (Melkonian *et al.*, 1999). Why are the triply palmitoylated influenza HA protein and dipalmitoylated T-cell integral membrane protein LAT (Zhang *et al.*, 1998), but not palmitoylated VSV G (Rose *et al.*, 1984) and transferrin receptors (Alvarez *et al.*, 1990), in lipid rafts/DRMs? Is it, as the authors suggested, due to the oligomerization potential of HA (trimers) that would "overcome" the TMD packing difficulties and allow efficient DRM association? Or some other unidentified reason? This has yet to be determined. It is here suggested that oligomerization (shown for the above integral membrane proteins), cholesterol association and palmitoylation may cooperate in localizing integral membrane proteins to DRMs/lipid rafts (Albert *et al.*, 1996; Thiele *et al.*, 2000).

Biophysical studies on lipid rafts have produced many interesting results. Jacobson and coworkers have shown that liquid-ordered domains can exist coupled in both leaflets of cellular membranes, potentially explaining the ability to have a lipid raft in the cytoplasmic leaflet which has a paucity of high T_m lipids like sphingolipids (Arni *et* *al.*, 1998; Dietrich *et al.*, 2001). However, these DRM areas in living cells were identified as transient confinement zones, due to their small and finite lifetime (tens of seconds) (Dietrich *et al.*, 2002). Can these structures account for the clustering of signal transducing molecules? This remains to be determined. To date, there is no direct evidence for the existence of sphingolipid-, cholesterol-containing l_o phase domains in living cells (Muniz and Riezman, 2000; Brown, 2001).

The issue of detergent insolubility as a criteria for raft association has its drawbacks. While 1% TX-100 has been traditionally used to generate DRMs, some reports utilize lower TX-100 concentrations to "enrich" their protein of interest in DRMs (Field *et al.*, 1997; Arni *et al.*, 1998). Many reports utilize the phospholipid dipalmitoylphosphatidylcholine (DPPC) in studies involving isolation of DRMs (Schroeder *et al.*, 1994; Utsumi *et al.*, 1996; Ahmed *et al.*, 1997; Ge *et al.*, 1999; Wang *et al.*, 2001a). This high T_m lipid is a rare phospholipid in cellular membranes, and as such may generate an artifact in partitioning due to its high propensity to generate a liquid-ordered membrane phase (Pike *et al.*, 2002). It is surprising that many lipid raft studies in the literature make use of this lipid species, yet its physiological relevance has yet to be determined. Also, membrane association does not equate to DRM localization, as exemplified by monopalmitoylated GAP-43 mutants, which are still partially membrane associated but lack DRM association (Arni *et al.*, 1998).

DRMs are believed to be generated only in membranes with potential to form them, such as endosomes, PM, Golgi, and TGN. The ER, due to its relatively low cholesterol and SL content, was not believed to contain DRMs (Brown and London, 1998b, a). However, Sevlever and coworkers assessed biochemically the subcellular distribution of DRMs, and found that ER fractions contained DRMs with associated GPI precursors and GPI-anchored proteins (Sevlever *et al.*, 1999). Whether this observation was the result of imperfect fractionation procedures or other artifact and its relevance to acylated or prenylated proteins are not known.

Acylation-dependent localization may be mediated solely by affinity of dually acylated or acylated plus polybasic domain containing proteins with bilayer regions of certain composition (*i.e.* high cholesterol, sphingolipid, polyanionic phospholipids, *etc.*). Conclusive demonstration of the existence of lipid rafts in living cells will be needed to prove that these entities in fact exist prior to processing of cells to generate lipid raft domains.

4.12 GENERAL MODEL OF N-TERMINALLY ACYLATED PROTEIN TRAFFICKING AND LOCALIZATION

Our comparison of different N-terminal membrane-association signals attached to a reporter protein has led to significant reproducible differences that can be grouped into three motifs: 1) dual acylation (myristoylation plus palmitoylation), 2) myristoylation plus polybasic domain, and 3) dipalmitoylation plus short polybasic domain. Such motifs induce subcellular localization, trafficking, membrane avidity, and regulation differences in the proteins that possess them.

Myristoylation is a permanent modification that provides the modified protein the ability to "dock" transiently throughout the bulk of endomembranes (mostly the widespread cellular ER network), which consists of a low-affinity reservoir of hydrophobic membrane that could act to dissipate the protein throughout the cell. This transient, continuous sampling (since the half-life of myristoylated peptides bound to membranes is on the order of seconds) would continue until a productive interaction with a PAT and an appropriate secondary signal such as palmitoylation would "lock" the protein into a particular membrane. Unlike Ras proteins, where significant processing occurs on the ER (proteolysis and carboxymethylation), N-terminally acylated proteins do not have equivalent processing steps. Dually acylated (myristoylation plus palmitoylation) proteins traffic to the plasma membrane via an uncharacterized mechanism. We saw no differences in the localization or fractionation of our FynGFP, $G\alpha_o$GFP, and LckGFP chimeras. However, it is interesting to note that Fyn, myristoylated and dipalmitoylated at Cys3 and Cys6 and a net charge of +2 in the first ten amino acids, traffics rapidly to the PM (< 5 min) (van't Hof and Resh, 1997). Similarly, a $G\alpha_{o10}$Fyn construct, with a net charge of –1 in the first 10 amino acids, trafficked rapidly. In contrast, Lck, a related NRTK, myristoylated and palmitoylated at Cys3 and 5, has a net charge of –2, and has been suggested to traffic via the exocytic pathway. When expressed in the same stably transfected cell system (NIH-3T3 cells), the differences in trafficking remained, with Fyn membrane associated in < 5 min, and Lck only 50% associated at 10 min, taking 30-40 min for complete association (Bijlmakers and Marsh, 1999). Can the difference in charge explain the differences in trafficking of the two proteins? It appears from the Gα_{o10}Fyn construct that a simple charge difference likely does not explain the trafficking differences, and suggests that other interactions must be occurring to produce the differences noted. Further work is needed to solve this mystery. Again, because we saw no differences in localization amongst our constructs, N-terminal myristoylation and palmitoylation may represent a default mechanism for membrane association, and that an overlay of protein-mediated or other interactions could drastically alter the trafficking of the protein.

Newly synthesized myristoylated and polybasic domain proteins such as Src traffic more slowly than myristoylated plus palmitoylated proteins (van't Hof and Resh, 1997; Wolven *et al.*, 1997). In our system, Src$_{16}$GFP localization was similar to that of myristoylated and palmitoylated GFP constructs, implying that these two motifs may be functionally redundant at the level of membrane localization. It is presumed that cotranslational myristoylation would promote a transient affinity for membranes first, but the role of the polybasic domain in this context is not known. We can speculate that ER-associated Src$_{16}$GFP would seek out a membrane environment that was favourable electrostatically. This charge affinity may prevent rapid sampling of ER membranes and redirect cytosolic myristoylated proteins onto particular endosomes (endosomal membranes are the most negatively charged of all membranes), with subsequent trafficking via these vesicles to the PM required (Cavenagh *et al.*, 1996). It could be that electrostatic-mediated retention at the plasma membrane/endosomes takes longer, by requiring clustering of anionic phospholipids, which may take a finite period of time to become physiologically significant. Unfortunately, the characterization of the role of electrostatic interactions in membrane association remains incomplete, and we can only speculate on the interactions of these different signals in membrane association and trafficking. In contrast to Fyn and Gα_o, which were >90% membrane–bound after 5 min, Src took upwards of two hours to reach steady state. A mutant Src with introduction of a Cys at position 3 and subsequent palmitoylation selectively conferred rapid membrane binding to Src (van't Hof and Resh, 1997). Thus, the polybasic domain of Src does not impede the trafficking to the PM if dual acylation is present at the N-terminus.

Paradoxically, rapid PM association of farnesylated, polybasic domain containing K-Ras4B in a microtubule-dependent fashion has been reported (Choy *et al.*, 1999; Apolloni *et al.*, 2000; Prior and Hancock, 2001; Prior *et al.*, 2001). Why the difference between these two proteins, which possess signals of apparent equivalent membrane associating potential, is an enigma. Perhaps the solution resides in the branched structures of the isoprenoid moiety *vs.* the linear extended structure of the saturated fatty acid.

Dipalmitoylated GAP-43 also traffics to the PM more slowly than myristoylated plus palmitoylated proteins. GAP-43 took upwards of two hours to reach steady state, showing a higher membrane-bound fraction than Src (90% *vs.* 75%) (van't Hof and Resh, 1997). Since GAP-43 does not contain cotranslational myristoylation, it must use another perhaps slower mechanism (protein-protein interaction, hydrophobic peptide sequence inserting into the bilayer, electrostatic interaction, *etc.*) to move from the cytosol after ribosomal translation and bind to the ERGIC/Golgi area where the presumptive PAT that palmitoylates it is located (McLaughlin and Denny, 1999). It has been suggested that the palmitoylation of GAP-43 occurs early in its biosynthetic life (Skene and Virag, 1989). GAP-43 also contains three basic amino acid residues in its N-terminal sequence, and their role in membrane binding is controversial, with one report demonstrating their importance, and another report finding no alteration in membrane association upon their mutation (Liu *et al.*, 1993, 1994). It is possible that it, like Src, utilizes electrostatic interaction plus acylation as its trafficking mechanism.

By analogy, the trafficking of C-terminal lipidated Ras proteins has been studied extensively (Choy *et al.*, 1999; Apolloni *et al.*, 2000; Prior and Hancock, 2001; Prior *et al.*, 2001). In contrast to rapid PM association of K-Ras4B in a microtubule-dependent fashion, H- and N-Ras trafficking took up to 2 h to complete, going through ER, Golgi, and secretory vesicles (Thissen *et al.*, 1997; Magee and Marshall, 1999). It is believed that K-Ras4B was able to bypass the Golgi or interact very transiently with, and move to the PM via a non-vesicular mechanism.

A general model of N-terminal protein acylation biogenesis, trafficking and ultimate localization is shown in **Figure 4.1**. This model summarizes knowledge from our study as well as from the literature. Once associated with these membranes, it is likely that these proteins associate with the lipid raft fraction in a detergent-sensitive way,

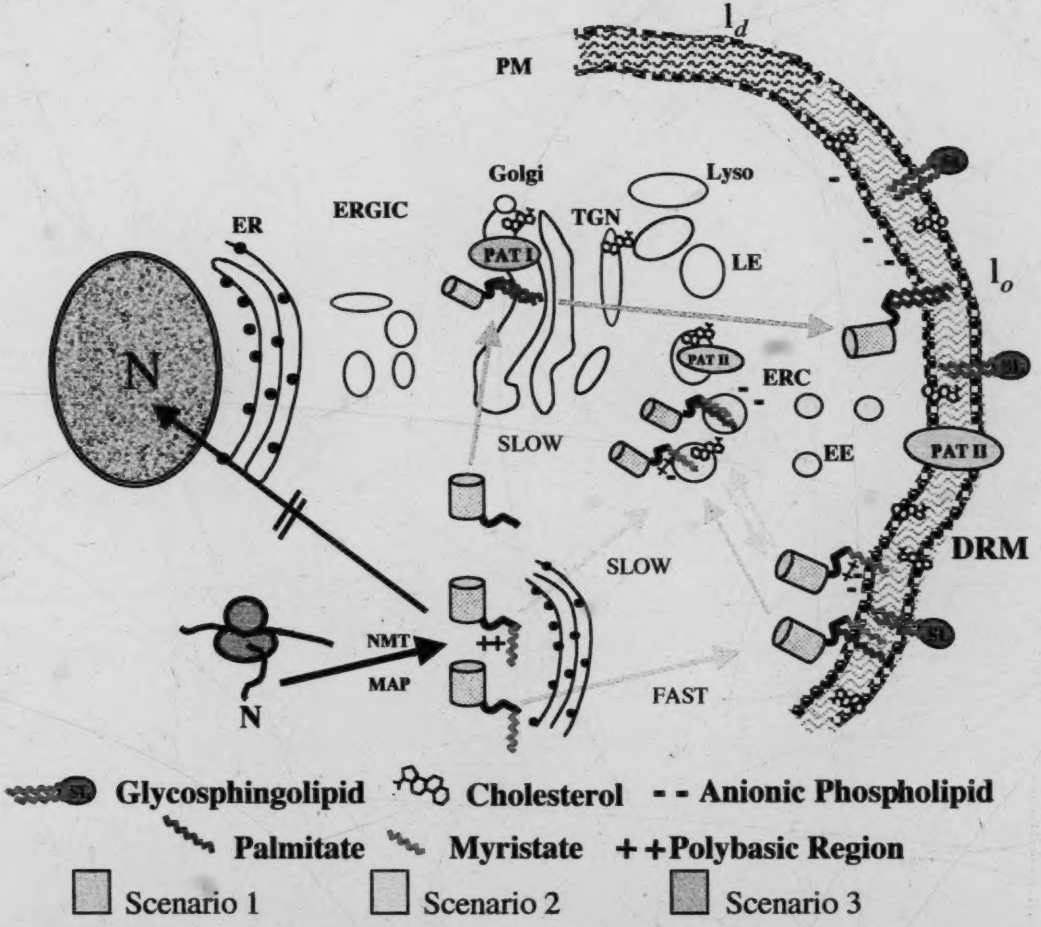
l_d
PM
Golgi
Lyso
ERGIC
ER
TGN
PAT I
LE
l_o
N
PAT II
ERC
SLOW
EE
PAT II
DRM
SLOW
NMT
++
MAP
N
FAST
Glycosphingolipid
Cholesterol
- - Anionic Phospholipid
Palmitate
Myristate
+ +Polybasic Region
Scenario 1
Scenario 2
Scenario 3

Figure 4.1. General Summary Model of N-Terminal Protein Acylation, Biogenesis, and Trafficking. Following synthesis on soluble ribosomes (blue, lower left), N-terminally acylated proteins appear to follow three paths to membrane association. Scenario 1 (green) depicts the path followed by a myristoylated and palmitoylated protein. Following cotranslational myristoylation, sampling of ER membranes rapidly leads to interaction with a membrane-bound PAT (PAT II) at the PM. Recycling back into endosomes is believed to occur. Scenario 2 (blue) depicts the path followed by a myristoylated plus polybasic region protein. Slower trafficking may be due to the polybasic domain restricting mobility of the chimera, or alternatively induce association with an endomembrane compartment. This may be due to interactions with anionic phospholipids, highly enriched in endosomes and the PM. For both Scenario 1 and 2 proteins, myristoylation prevents nuclear localization from occuring (arrow). Scenario 3 (orange) depicts the path followed by a dipalmitoylated GFP chimera. As the chimera contains no obvious membrane associating lipid signals, a primary sequence motif, possibly the small polybasic domain, promotes interaction with a Golgi PAT (PAT I). This is followed by trafficking through the secretory pathway to the PM. Both Scenario 2 and 3 proteins have been shown to traffic more slowly than Scenario 1 proteins in the literature. Ultimately, the acylated proteins arrive at the PM/endosomes, where localization to the immediate periphery of lipid rafts or localization to lipid rafts in a detergent-sensitive fashion is believed to occur (see **Figure 3.13**).

enriching the proteins in the vicinity of detergent-insoluble lipid raft cores and facilitating protein-protein interactions mediating transfer to the DRM cores (**Figure 3.9**).

4.13 CONCLUSIONS

This study of the functional roles of N-terminal protein acylation has identified three partially characterized membrane association signal combinations that lead to intracellular localization with PM, endosomes and Golgi. This localization is mediated by partitioning into membrane domains enriched in free cholesterol, sphingolipids, and anionic/saturated phospholipids. Based on our detergent solubility data, we propose that acylated proteins localize to the immediate vicinity of DRMs/lipid rafts. DRM association appears to be mediated by protein-protein interactions, common amongst signal transducing proteins. These supramolecular clusters are believed to enhance the transmission and fidelity of signal transduction processes. This work, by virtue of its [illegible] and gain-of-function analyses and addition of short N-terminal signals in the absence of protein-protein interaction domains, lends greater strength to the conclusions presented here on the intrinsic role of N-terminal protein fatty acylation in subcellular localization. Overall, we show that biophysical properties of both acylated proteins and acceptor membranes represent an exquisite means of localizing proteins intracellularly.

4.14 FUTURE DIRECTIONS

It is predicted that the understanding of protein lipidation as it relates to subcellular localization will ultimately be linked to advances and developments in the field of biophysics. This idea is not novel, as it was mentioned in a review some 5 years ago (Bhatnagar and Gordon, 1997). Unfortunately, the progress has been constant but slow, reflecting some of the inherent difficulties present when studying protein lipidation. While significant contributions have been made in the area of biophysics as it pertains to protein lipidation, conflicting reports and theoretical extrapolations have made interpretation less clear.

Our data suggest a few questions worth pursuing in the future and would include:

1) How does the nucleus exclude myristoylated proteins? Is the fact that there are no

apparent acylated GFP chimeras in the nucleus due to the absence of membranes in the nucleus or is there a specific mechanism excluding acylated proteins from the nucleus? We could assess this by carrying out microinjection studies with myristoylated proteins made in bacteria being introduced into the nucleus and cytoplasm of COS-7 cells. Then, we could assess how the nucleus handles such entities by measuring the rates of exit from the nucleus between differentially acylated and nonacylated GFPs. In such experiments, it could be interesting to see if there is a rapid association of myristoylated proteins with the endosomal recycling compartment prior to PM localization.

2) What are the kinetics of membrane association and trafficking of fatty acylated proteins? Do acylated proteins go to the PM first, and then are recycled to endosomes, or are they localized to endosomes first and then transported to the PM? One could assess this by microinjection of acylated GFPs into cells and tracking their movement in real time. Pulse-chase analysis using radiolabeled fatty acids and amino acids would also be useful in tracking newly synthesized molecules as they emerge from the ribosomal surface. Finally, the use of inhibitors such as cycloheximide, aluminum fluoride, tunicamycin, BFA, nocodazole, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and monensin, or dominant-negative dynamin or MβCD to prevent endocytosis could help elucidate this as well. Another useful technique to solve this problem would be fluorescence recovery after photobleaching (FRAP). By irradiating a small region of intracellular endosomal membranes and the plasma membrane, the recovery characteristics could provide clues as to the operative trafficking mechanism.

3) Do endosomes contain PAT? We could isolate endosomes using immunoaffinity purification or alternatively free flow electrophoresis. With large scale isolation purification, 2-D electrophoresis, and microsequencing, a systematic analysis of the proteins contained within these structures could be performed. I would assess for presence of acylated proteins, ACS, acyl-CoAs, and the interaction of ACBP with endosomes. Relating to the difficulties of characterizing and cloning PAT, I propose

the question, "Does the presence of an ACS alone mediate protein palmitoylation by providing substrate at a site competent for membrane trapping according to the Shahinian and Silvius model?" There is precedence for endosomal association of ACS, in GLUT4 glucose transporter-containing vesicles that are competent for protein palmitoylation following immunoadsorption and provision of palmitate, ATP, and CoA.

4) Does the manipulation of a polybasic region downstream from acylation sites lead to alternate trafficking rates for proteins so modified? Many dually acylated (myristate plus palmitate: Yes, Fyn, Gα_o) proteins have been reported to traffic rapidly from their site of synthesis to the PM. Myristate plus polybasic region proteins (Src) traffic slowly to the PM, and introduction of a second acylation (palmitoylation) site to Src induces rapid trafficking. Does the enhancement of polybasic domain size impact on the electrostatic interactions and prevent/augment palmitoylation, and does it reroute the protein from trafficking rapidly to the plasma membrane to more slowly like Src? These constructs will be followed by pulse-chase analysis and compared with rates of membrane association in the literature. It is hoped that the membrane binding implications of acylation and electrostatic interactions can be further characterized, to see if they cause different cellular trafficking pathways to be used, and to observe what the outcome is when both are present: do they synergize or compete for these pathways?

5) Do C-terminal K-Ras chimeric proteins traffic in a similar fashion to Src GFP chimeras? Both proteins contain similar lipid plus polybasic membrane associating signals: farnesyl plus polybasic for K-Ras, myristate plus polybasic for Src. By coexpression of K-Ras and Src sequences appended to different reporter proteins (GFP and other color fluorescent proteins: blue, cyan, and yellow), the subcellular fates of these two proteins could be followed. Using live cell confocal microscopy, the initial synthesis and trafficking could be monitored in real time. Additionally, fluorescence energy resonance transfer (FRET) could be utilized to assess the possible close interaction of these two proteins in a common trafficking pathway. It

will be interesting to ascertain why, in the literature, there is a discrepancy in the trafficking times of these two proteins to the plasma membrane (van't Hof and Resh, 1997; Choy *et al.*, 1999; Apolloni *et al.*, 2000; Prior *et al.*, 2001).

6) What primary sequence domains mediating protein-protein interactions in Yes NRTK allow for the fractionation of the full-length protein to DRMs? In our study, an 11 amino acid N-terminal portion of Yes did not fractionate into DRMs when appended to GFP, but the full-length protein did. As was done for ecNOS (Prabhakar *et al.*, 2000), using chimeric protein and PCR technology, the study of the minimal requirements for DRM/lipid raft association will be conducted with the Yes NRTK. With YesGFP already created, crystal structures of related NRTK members Src and Hck reported in the literature (Sicheri and Kuriyan, 1997; Sicheri *et al.*, 1997; Xu *et al.*, 1997), and a cDNA of the full-length Yes protein in our laboratory, the breakdown of what mediates association with DRMs could be experimentally determined.

CHAPTER FIVE

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CHAPTER 6

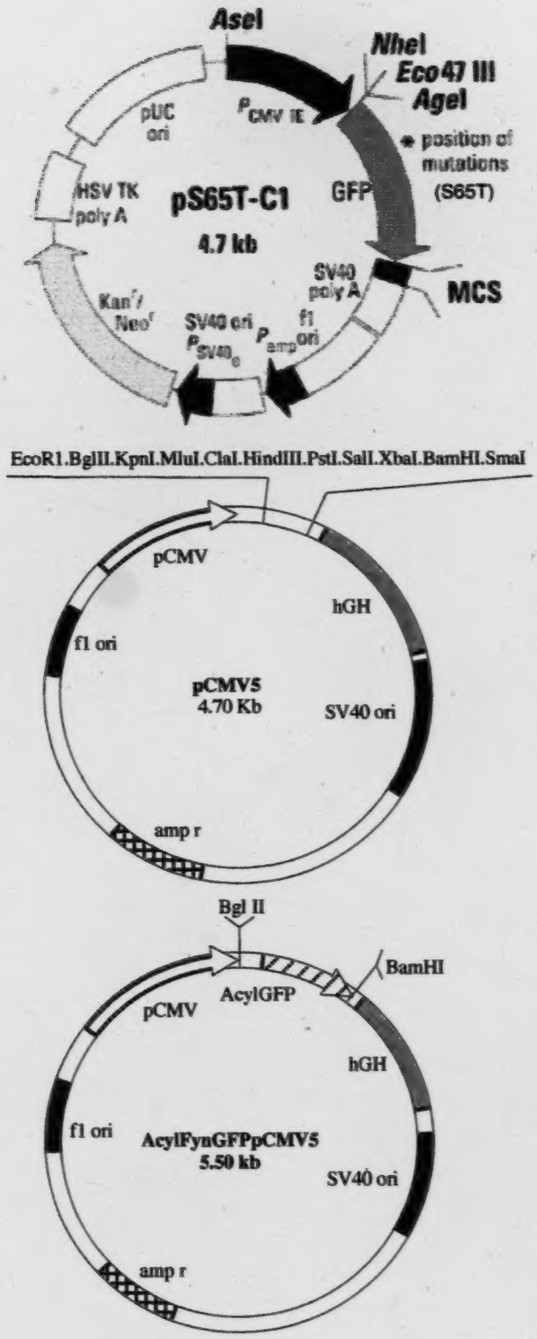
APPENDICES

6.1 BACTERIAL STRAINS AND MEDIA

Escherichia coli DH5α was obtained from laboratory stocks and grown in Luria-Bertani (LB) medium at 37°C (Hanahan, 1983; Sambrook *et al.*, 1989). Transformed *E. coli* DH5α was grown in LB medium supplemented with either 50 µg/ml kanamycin or 100 µg/ml ampicillin (Sigma). Solid media was prepared by the addition of 1.5 % bacto-agar (Difco). Frozen stocks of *E. coli* were prepared by adding sterile glycerol to the cell suspension to a final concentration of 15%. Preparation of competent cells was as described with modifications (Hanahan, 1983). A 5 ml overnight starter culture of *E. coli* in Super Optimal Broth (SOB) medium was used to seed 500 ml of SOB and grown to OD_{600} 0.4-0.5. The culture was divided into two aliquots, centrifuged at 2600 x *g* and the pellets each resuspended in 25 ml of cold 0.1 M $CaCl_2$. The cell suspensions were chilled on ice for 30 minutes, centrifuged at 2600 x *g* for five minutes, and cell pellets were each resuspended in 5 ml 0.1 M $CaCl_2$/10% glycerol. Competent cells were aliquoted and frozen at -80°C.

6.2 PLASMIDS, DNA ISOLATION AND PURIFICATION

The pS65TGFP-C1 vector (**Figure 6.1**; Clontech, Palo Alto, CA) encodes a variant of WT GFP (Inouye and Tsuji, 1994; Prasher *et al.*, 1992). S65T GFP contains one amino acid substitution: Ser-65 to Thr. The S65T GFP variant produces a fluorescence that is five times more intense than that of wild-type GFP, and folds four times as quickly (Heim *et al.*, 1995). In pS65T-C1, the GFP-S65T coding sequences are downstream from the CMV immediate early promoter ($P_{CMV\ IE}$). The GFP stop codon has been replaced by a multiple cloning site (MCS). Genes cloned into this MCS will be expressed as fusions to the C-terminus of GFP-S65T if they are in the same reading frame as GFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the GFP-S65T gene direct proper processing of the 3' end of the GFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing SV40 T-antigen (COS-7 cells). A neomycin-resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex thymidine kinase gene, allows stably transfected eukaryotic


AseI
NheI
Eco47 III
AgeI
PCMV IE
pUC ori
* position of mutations (S65T)
GFP
HSV TK poly A
pS65T-C1
4.7 kb
SV40 poly A
MCS
Kanr/Neor
SV40 ori
PSV40e
Pamp
f1 ori
EcoR1.BglII.KpnI.MluI.ClaI.HindIII.PstI.SalI.XbaI.BamHI.SmaI
pCMV
hGH
f1 ori
pCMV5
4.70 Kb
SV40 ori
amp r
Bgl II
BamHI
AcylGFP
pCMV
hGH
f1 ori
AcylFynGFPpCMV5
5.50 kb
SV40 ori
amp r

GFP Reporter Protein Vector

COS cell Expression Vector

Vectors containing N-terminal Acylation Sequence GFP Fusions

Figure 6.1. Plasmids Used or Assembled in this Study.

cells to be selected using G418. A bacterial promoter upstream of this cassette (P_{amp}) confers kanamycin resistance in *E. coli*. The GFP-C1 backbone also provides a pUC19 origin of replication for propagation in *E. coli* and an f1 origin (f1 ori) for single-stranded DNA production in filamentous phage. An additional *Sna*BI site, not present in wild-type GFP, is linked to the GFP-S65T mutation in pS65T-C1 and can be used to distinguish the variant and wild-type GFP genes.

The various engineered N-terminal acylation sequences were fused to the cDNA of GFP by PCR and were subcloned into the pCMV5 mammalian expression vector (**Figure 6.1**) (Andersson *et al.*, 1989). This vector incorporates an SV40 origin of replication enabling propagation in SV40-and SV40 large T-transformed cell lines, and is especially efficient in COS cells. It also possesses a f1 origin of replication, the CMV promoter, the human growth hormone termination and poly-adenylation signals and an ampicillin resistance gene.

Plasmid DNA for PCR and sequencing was isolated from *E. coli* DH5α by modified alkaline lysis (Birnboim and Doly, 1979; Sambrook *et al.*, 1989), followed by purification on a glassmilk matrix (Vogelstein and Gillespie, 1979). Small-scale preparations (5-10 ml) were performed using the RPM Miniprep kit (Bio101), while large-scale preparations (≥ 1 L) were performed using the RPM 4G Mini Monster Kit (Bio101). All DNA solutions were prepared in Tris-EDTA (TE) buffer (10 mM Tris pH 8.0, 1 mM EDTA) and stored at -20°C.

6.3 OLIGONUCLEOTIDE DESIGN, PREPARATION, AND PLASMID CREATION

All primers (**Table 6.1**) were synthesized at the DNA Core Facility (Department of Biochemistry, University of Alberta) using the phosphoramidite method at the 40 nmol scale (Beaucage and Caruthers, 1981). Oligonucleotides were prepared by resuspending the purified oligonucleotide in 200 μl TE, heating to 65°C for ten minutes, then cooling on ice. The OD_{260} of an initial 1/100 dilution of oligonucleotide stock was determined using a quartz cuvette. The OD_{260} was multiplied by the dilution factor and the conversion factor 30 OD_{260}=1 mg/ml single-stranded DNA (Sambrook *et al.*, 1989) to determine the oligonucleotide concentration. The molecular weight of the oligonucleotide was then

Table 6.1: DNA Sequences of Primers Used in this Project

Oligonucleotide Name	Brief Description	Sequence
Sense Primers (5'→3')		
pET-BglII-T7-23S	Creates ***Bgl*II** restriction site before pET vector T7 promoter	5'-cggcgtagaggatcg**agatct**cg-3' (23-mer)
GFP-ATG-21S	For making fusions with GFP starting sequence	5'-atgagtaaaggagaagaactt-3' (21-mer)
GFP-BspHI-27S	Creates ***Bsp*HI** restriction site 5' to GFP start codon	5'-**cgcgtca**tgagtaaaggagaagaactt-3' (27-mer)
T7-GAP-77S	Creates pT7 3' + GAP-43$_{11}$ + Fyn$_7$ DNA fragment	5'-taacttttaagaaggagatataccatgctgtgctgc atgcgtcgtaccaaacaggttacaaaactgacggaggagagg-3' (77-mer)
SOET7-GFP-44S	Used to SOE BglII-pT7 directly onto GFP sequence	5'-taactttaagaaggagatataccatgagtaaaggagaagaactt-3' (44-mer)
T7GAP(C3,4S)-44S	Creates C3,4S mutation in GAP-43FynGFP	5'-taactttaagaaggagatataccatgctgtcctccatgcgtcgt-3' (44-mer)
Antisense Primers (3'→5')		
SOE-24AS	Used for SOE fusions with 3' end of pET T7 promoter	5'-tatatctccttcttaaagttaaac-3' (24-mer)
Src$_{16}$FynGFP-69AS	Addition of Arg15+16 to Src$_{14}$GFP construct	5'-aagttcttctcctttactxatacgttcttcggtcagtt tggtacgacggcgctggctggggtccttagg-3' (69-mer)
SOEFyn-GFP-42AS	Used to SOE GFP onto 3' end of Fyn constructs	5'-aagttcttctcctttactcatcctctcctccgtcagttttgt-3' (42-mer)
GFP-BamHI-30AS	Creates ***Bam*H I** restriction site 3' to GFP stop codon	5'-cgc**ggatcc**ttatttgtatagttcatccat-3' (30-mer)
Sequencing Primers		
pCMV5-21S	Used for sequencing ligated pCMV5 inserts, before polylinker	5'-tatagcagagctcgtttagtc-3' (21-mer)
pCMV5-21AS	Used for pCMV5 insert sequencing, reverse direction (3'-5')	5'-aacttccaaggccaggagagg-3' (21-mer)

Items in **Bold** refer to restriction enzymes and their consensus sequences
Abbreviations: SOE, splicing by overlap extension; GFP, green fluorescent protein; ATG: Met start codon; T7: bacterial T7 promoter

used to establish the molarity of the solution. Working solutions of 10 pmol/μl were prepared in TE, and all oligonucleotide solutions were stored at -20°C.

The method of splicing by overlap extension (SOE) (Horton *et al.*, 1989) was utilized to create fusion genes in which either wild-type or mutant N-terminal sequences of NRTKs Fyn, Yes, Lck, and Src, $G\alpha_o$, and GAP-43 were appended to the N-terminus of GFP (**Figure 6.2**). A number of plasmids with variants of these sequences were created and appended to Fyn PTK truncated after the SH2 domain by Dr. Berthiaume while completing a postdoctoral fellowship with Dr. Marilyn Resh (Memorial Sloan Kettering Cancer Center, New York, NY, USA). As such, an antisense primer (SOEFyn-GFP-42AS) encoding Fyn amino acid codons 12-18 and the first seven amino acid codons of GFP was used in PCR with the sense primer pET-*Bgl*II-T7-23S and various pET19b vectors containing WT and mutant Fyn, SrcFyn, $G\alpha_o$Fyn, and LckFyn sequences in a truncated FynSH432 protein vector (Alland *et al.*, 1994). This was done to create various pT7 promoter+N-terminal acylation sequence+GFP DNA fragments. A second DNA fragment was created by conducting PCR with the sense primer GFP-ATG-21S and antisense primer GFP-*Bam*HI-30AS, and pS65T-C1 GFP vector as template. This created a DNA fragment encoding the entire GFP sequence, and a 3' *Bam*HI restriction site. Then, SOE was performed by using the sense primer pET-*Bgl*II-T7-23S and antisense GFP-*Bam*HI-30AS with two DNA templates simultaneously, *Bgl*II-pT7-AcylFyn-GFP and GFP*Bam*HI. SOE created fusion fragments of approximately 900 kb, *Bgl*II-pT7-AcylSequence-Fyn_7GFP*Bam*HI, which were subcloned into the pCMV5 expression vector. This method of construction was used for Fyn, Yes, Lck, $G\alpha_o$, and Src, as well as mutants Fyn(C3,6S), Yes(C3S), Fyn(G2A), and Yes(G2A).

To create GAP-$43_{11}$$Fyn_7$GFP, the sense primer T7-GAP-77S was synthesized, and incubated with the antisense primer GFP-*Bam*HI-30AS and template vector Fyn_{18}GFPpCMV5. Subsequently, the sense primer pET-*Bgl*II-T7-23S was incubated with SOE-24AS and pET19bFynSH432 vector, to create a small DNA fragment, *Bgl*II-pT7. SOE was then performed, using sense primer pET-*Bgl*II-T7-23S and antisense primer GFP-*Bam*HI-30AS, with simultaneous templates *Bgl*II-pT7 and pT7-GAP-$43_{12}$$Fyn_7$GFP*Bam*HI. This created a 900 kb fragment, *Bgl*II-pT7-GAP-

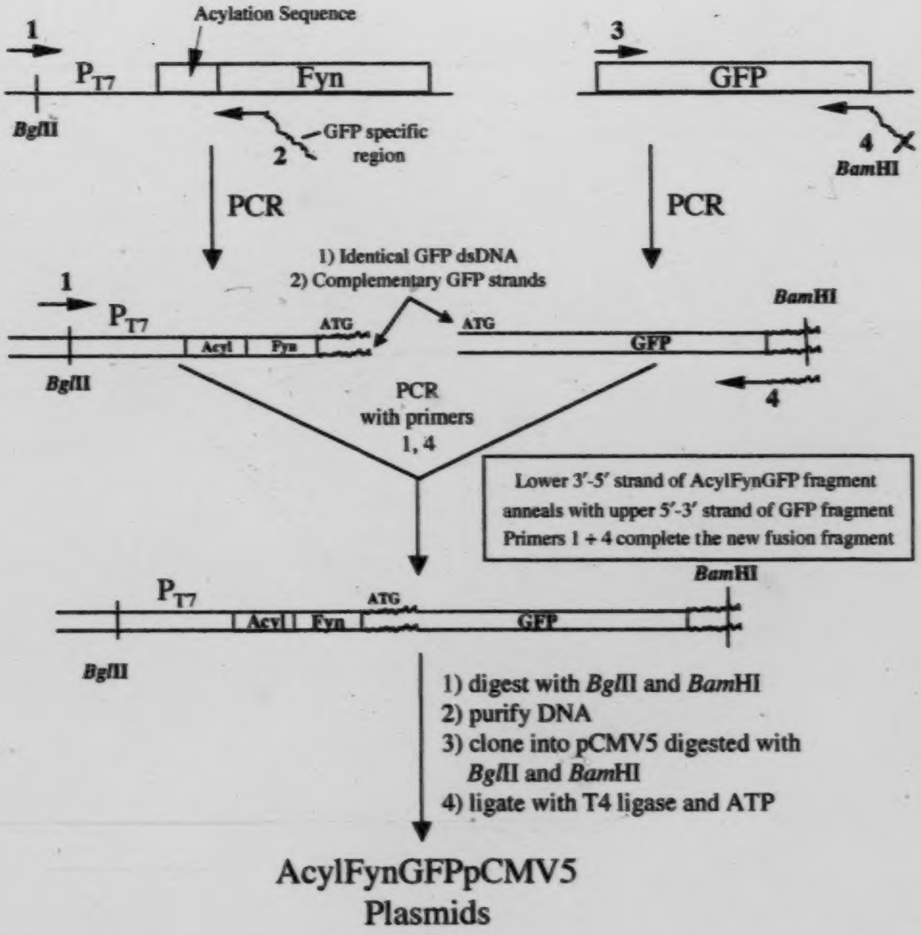

Acylation Sequence
1
P_{T7}
Fyn
BglII
GFP specific region
2
3
GFP
4
BamHI
PCR
PCR
1) Identical GFP dsDNA
2) Complementary GFP strands
1
P_{T7}
ATG
ATG
Acyl
Fyn
BglII
GFP
BamHI
4
PCR with primers 1, 4
Lower 3′-5′ strand of AcylFynGFP fragment anneals with upper 5′-3′ strand of GFP fragment
Primers 1 + 4 complete the new fusion fragment
P_{T7}
ATG
Acyl
Fyn
GFP
BamHI
BglII
1) digest with BglII and BamHI
2) purify DNA
3) clone into pCMV5 digested with BglII and BamHI
4) ligate with T4 ligase and ATP
AcylFynGFPpCMV5 Plasmids

Figure 6.2. Splicing by overlap extension (SOE) method for generating chimeric GFP constructs. Wild type and mutant N-terminal acylation sequences were appended to the N-terminus of GFP. To do so, oligonucleotide sense primer 1 (encoding a *Bgl*II restriction site) and fusion antisense primer 2 (containing DNA sequence from Fyn and GFP) are incubated with various DNA Fyn template plasmids (upper right), generating a *Bgl*II-AcylFynGFP DNA fragment. Fusion sense primer 3 (containing DNA sequence from the start of GFP) and antisense primer 4 (containing a *Bam*HI restriction site) are incubated with the GFP cDNA in a separate PCR reaction, generating the GFP-*Bam*HI DNA fragment. The two products are combined with sense primer 1 and antisense primer 4, allowing the annealing of complementary ends of *Bgl*II-AcylFynGFP and GFP-*Bam*HI. This generates the large *Bgl*II-AcylFynGFP-*Bam*HI fragment. Following digestion with *Bgl*II and *Bam*HI, generating overhanging sticky ends, and purification, the fragment is subcloned into pCMV5 expression vector digested in the same fashion. The vector and fragment are ligated with T4 DNA ligase, and ready for large scale preparation and subsequent cell culture transfection.

43_{12}Fyn$_7$GFP*Bam*HI, which was then subcloned into pCMV5.

To create the Cys→Ser mutant of GAP-43, GAP-43 (C3,4S)FynGFP, the sense primer, T7GAP(C3,4S)-44S was created. This generates a DNA fragment, pT7-GAP-43(C3,4S)$_{11}$Fyn$_7$GFP*Bam*HI when incubated with the antisense primer GFP-*Bam*HI-30AS, and GAP-43$_{11}$Fyn$_7$GFPpCMV5 vector template. SOE was then performed with sense primer pET-*Bgl*II-T7-23S, antisense primer GFP-*Bam*HI-30AS, and simultaneous templates *Bgl*II-pT7 and pT7-GAP-43(C3,4S)$_{11}$Fyn$_7$GFP*Bam*HI. The resultant full fragment, *Bgl*II-pT7-GAP-43(C3,4S)$_{11}$Fyn$_7$GFP*Bam*HI, was then ready for subcloning.

Following DNA sequencing of the "apparent" Src$_{11}$Fyn$_7$GFPpCMV5 vector, it was noted that a PCR error had been present in the template vector obtained from New York. This had inadvertently created a slightly altered SrcFynGFP construct with 14 amino acids residues of Src (1-14) and 4 amino acids residues of Fyn (15-18), followed by GFP. As this mutant chimera possesses a net charge of only +2 in its N-terminal region, a second Src construct, Src$_{16}$Fyn$_7$GFP, was created which possessed a more extensive polybasic domain (net charge +5). This was performed by adding two WT-sequence Arg residues, as well as the full TKLTEER Fyn sequence. To enable this, an antisense primer, Src$_{16}$FynGFP-69AS was synthesized, combining seven original amino acid codons of Src (8-14), the additional Src polybasic amino acid codons (15-16), the seven amino acid codons of Fyn (TKLTEER), and the first seven amino acid codons of GFP (MSKGEEL). When incubated with the sense primer pET-*Bgl*II-T7-23S and Src$_{14}$Fyn$_4$GFPpCMV5 vector, the DNA fragment, *Bgl*II-pT7-Src$_{16}$Fyn$_7$GFP was generated. SOE was then performed, using primers pET-*Bgl*II-T7-23S, GFP-*Bam*HI-30AS, and templates *Bgl*II-pT7-Src$_{16}$Fyn$_7$GFP and GFP*Bam*HI. The resulting final DNA fragment, *Bgl*II-pT7-Src$_{16}$Fyn$_7$GFP*Bam*HI, was then subcloned into pCMV5.

6.4 POLYMERASE CHAIN REACTION

Polymerase chain reactions (PCR) were conducted in 50 µl volumes and included 100 ng of template DNA, 20-40 pmol of each sense and antisense oligonucleotide primer constituting the appropriate primer pair, 5 µl of 10X ThermoPol reaction buffer (New England Biolabs; NEB), 1 U Vent DNA polymerase (NEB) and 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP; Promega). Amplification was allowed to proceed for thirty cycles, with each cycle incorporating successive steps at 94°C for 30 seconds, 51°C for 30 seconds, and 72°C for 90 seconds. The first cycle was preceded by an initial denaturing step of 94°C for five minutes, and the last cycle was followed by a final elongation step of 72°C for ten minutes.

6.5 GENERAL CLONING METHODS

Restriction digests of pCMV5 vector and N-terminal acylation sequence GFP amplification fragments were carried out at the appropriate temperature(s) for 1-2 hours in a final volume of 50 µl using restriction enzymes and 10X buffers obtained from NEB. Bovine serum albumin (BSA) was added to a final concentration of 400 µg/ml.

Digested pCMV5 vector was dephosphorylated using calf intestinal alkaline phosphatase (NEB) in two stages; 2.5 U of enzyme was added to the digestion mixture and incubated at 37°C for 30 minutes, then an additional 2.5 U of enzyme was added and the mixture was incubated for a further 30 minutes. Reactions were terminated by heating the reaction mixture at 75°C. The dephosphorylated vector was then gel purified.

Agarose and acrylamide gel purification of amplified PCR products, digested inserts and pCMV5 vector used in cloning was done using a modified "crush and soak" method (Sambrook *et al.*, 1989) adapted from Maxam and Gilbert (Maxam and Gilbert, 1977). After separation of the DNA on 6% polyacrylamide (digested vector) or 0.8% agarose (PCR products or digested inserts) and staining with ethidium bromide, the appropriate bands were excised and crushed in an Eppendorf tube using a Kontes pellet pestle and 25 µl TE. The mixture was incubated at 37°C for one hour, then centrifuged through a Spin-X tube (Costar) at 13 200 x *g* to separate the gel matrix from the DNA filtrate.

Directional ligation of the digested AcylFynGFP inserts into digested pCMV5 was carried out in a 50 µl mixture containing an approximate 10:1 insert to vector molar ratio, 5 µl of 10X ligase buffer (Gibco) and 1 U of T4 DNA ligase (Gibco). Ligation was carried out overnight at 16°C. Competent *E. coli* DH5α were transformed with ligation mixture volumes corresponding to 1 ng and 5 ng of vector DNA.

6.6 GENERATION OF N-TERMINAL SEQUENCE-GFP CHIMERA VECTORS

To create the chimeric GFP vectors, the PCR amplification fragments of approximately 0.9 Kbp were isolated from 6% acrylamide gel, digested with *Bgl*II and *Bam*HI to generate cohesive ends, then repurified from acrylamide, as previously described. The receiving pCMV5 vector was also digested with *Bgl*II and *Bam*HI to generate cohesive ends and purified from 0.8% agarose. Approximated quantities of the individual inserts and vectors, corresponding to a 10:1 insert to vector ratio, were ligated as described in Appendix Section 6.5 and the mixture transformed into *E. coli* DH5α. After confirmatory digestion with *Bgl*II and *Bam*HI, a single clone for each construct was chosen for dideoxy chain termination based-sequencing (Sanger *et al.*, 1977) at the DNA Core Facility (Department of Biochemistry, University of Alberta). The final construct (pCMV5AcylFynGFP) in vector form appears in **Figure 6.1**, and a 3-D schematic model showing the crystal structure of GFP with the linker region and variable N-terminal acylation sequences is shown in **Figure 6.3**.

6.7 RADIOIODINATION OF PALMITATE

Preparation of the iodopalmitate analogue was carried out as described by Berthiaume *et al.* without the HPLC purification step (Berthiaume *et al.*, 1995). [^{125}I]NaI (2-3 Ci/mmol SA) was purchased from Amersham Pharmacia. Iodopalmitic acid was a gift from Dr. M. Resh, Sloan-Kettering Cancer Center, New York, NY. This was dissolved in acetone to produce a 10 mM stock and stored at -20°C. In a glass reaction vessel, 200 μl (2 μmol) of iodopalmitate stock was evaporated and treated with 3 μl glacial acetic acid. The [^{125}I]NaI vial was rinsed twice with 150 μl of acetone, and each rinse transferred to the reaction vessel. The vessel was capped with a rubber septum, and a charcoal trap inserted. The radioiodination reaction proceeded at 55°C for 16 hours. Distilled water (0.5 ml) was added to the reaction tube and the aqueous layer extracted twice with 1.2 ml of chloroform, which was transferred to a clean vial and evaporated under nitrogen. The residue was resuspended in 1070 μl of 95% ethanol and ten 100 μl aliquots were dispensed into glass vials, dried under nitrogen and stored at -20°C. The remainder of the original suspension was diluted and counted in a scintillation counter, with typical specific activity of the final [^{125}I]iodopalmitate preparation at 2-3 Ci/mmol. Each vial of [^{125}I]iodopalmitate was resuspended in 15-20 μl of 95% ethanol before use. Iodopalmitate for experimentation was prepared by Zhao Yang, technician in the

Berthiaume Lab.

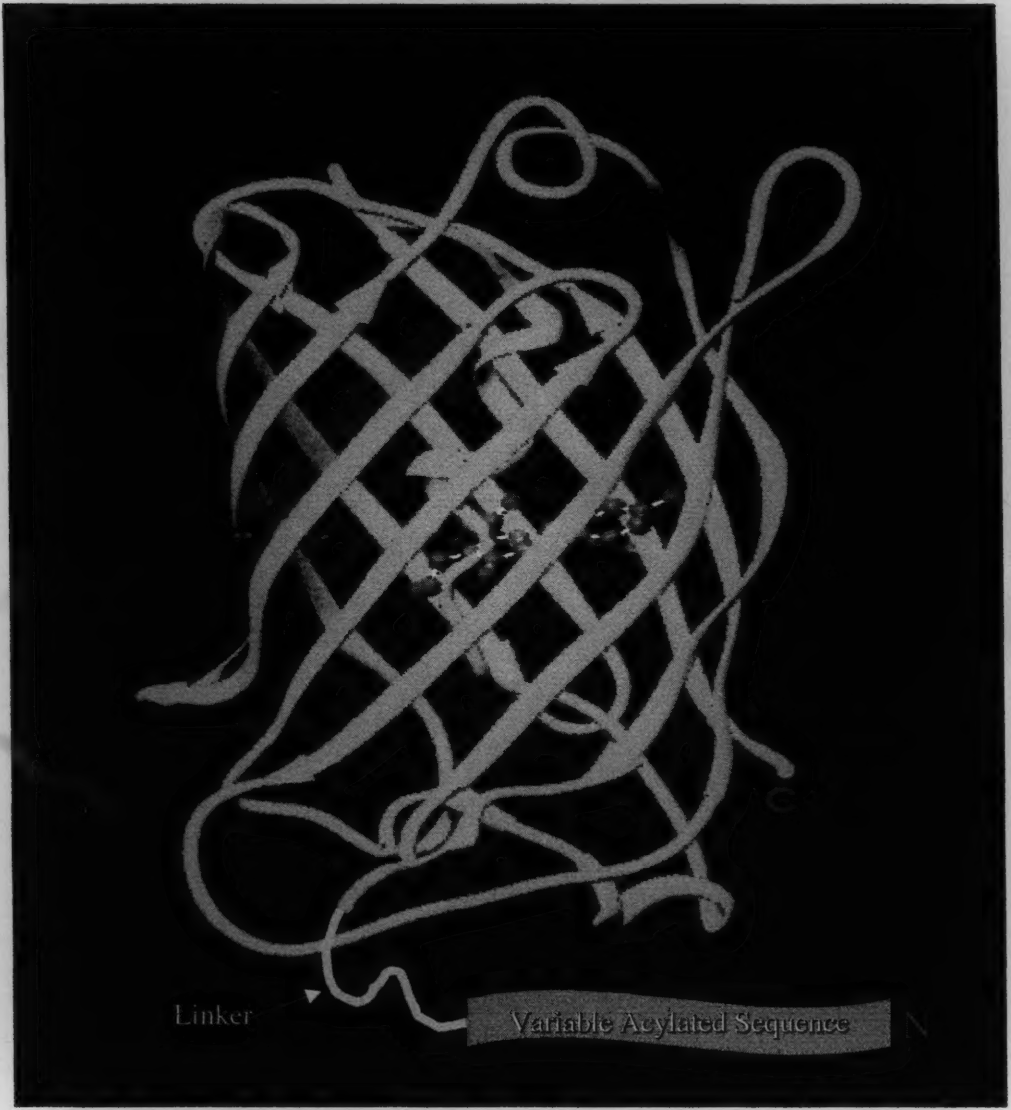


Figure 6.3. 3-D structure of green fluorescent protein from *Aequorea victoria*. Shown is the 11 β-strand barrel motif with the autofluorescent structure, formed by an intramolecular cyclization, buried within the hydrophobic core of the molecule. A seven amino acid linker (TKLTEER) (cyan) was added between the GFP reporter protein and the variable acylation sequence (blue) to facilitate covalent modification by lipid via accessibility to the cellular enzymatic machinery and acyl-CoA pools. The 3-D structure model was adapted from a previous study (Ormo *et al.*, 1996).

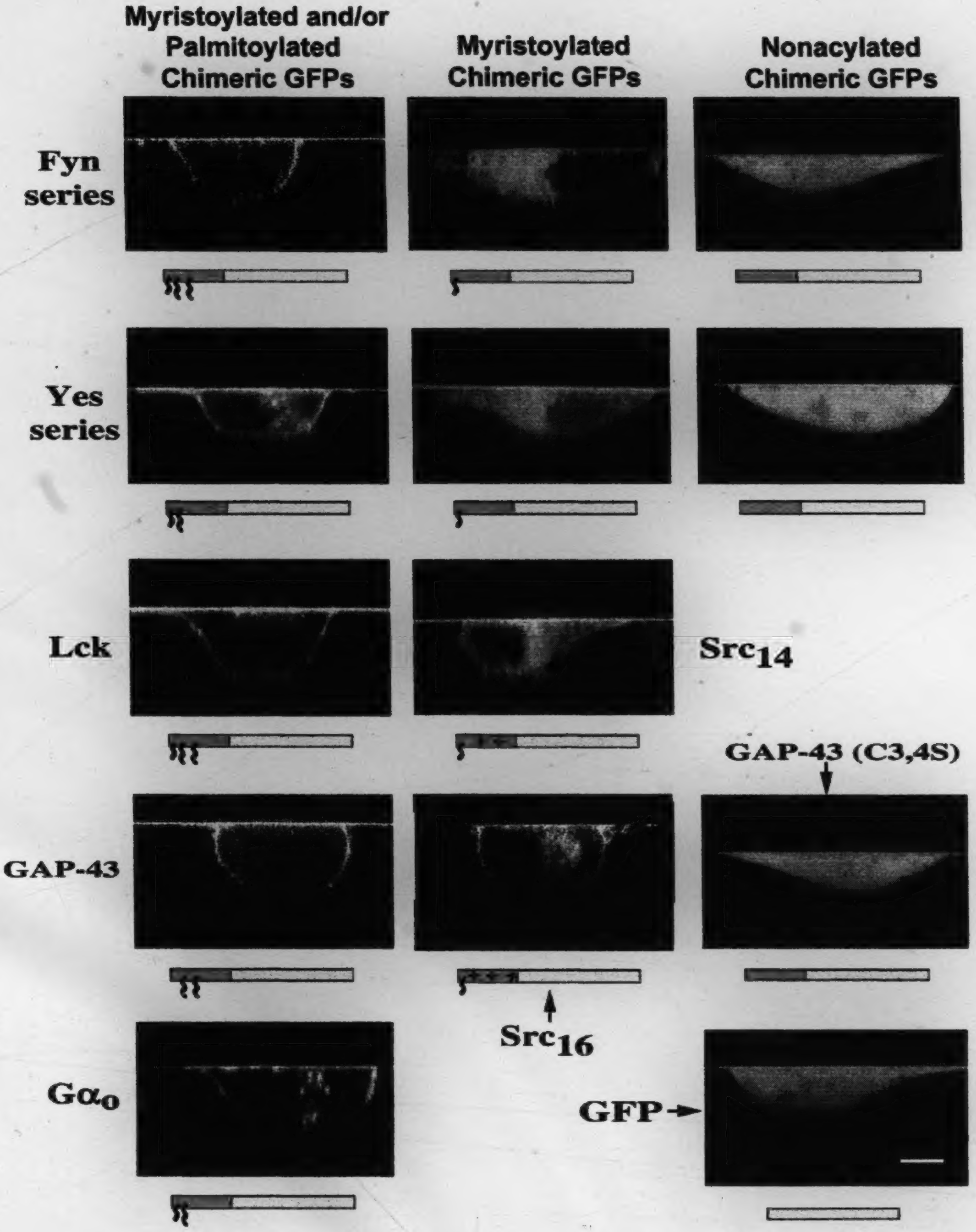

Myristoylated and/or Palmitoylated Chimeric GFPs
Myristoylated Chimeric GFPs
Nonacylated Chimeric GFPs
Fyn series
Yes series
Lck
Src$_{14}$
GAP-43 (C3,4S)
GAP-43
Src$_{16}$
Gα$_o$
GFP

Figure 6.4. Subcellular localization of various N-terminal fatty acylated GFP chimeras and mutants in living COS-7 cells in the XZ plane. GFP fluorescence of COS-7 cells expressing various GFP chimeras. Intrinsic GFP fluorescence was detected by confocal laser scanning microscopy. Note enrichment of fluoresence at the edge of cells and where the cells attach to the substratum in cells expressing dually acylated or acylated and polybasic GFP chimeras. Bar, 10 μm.

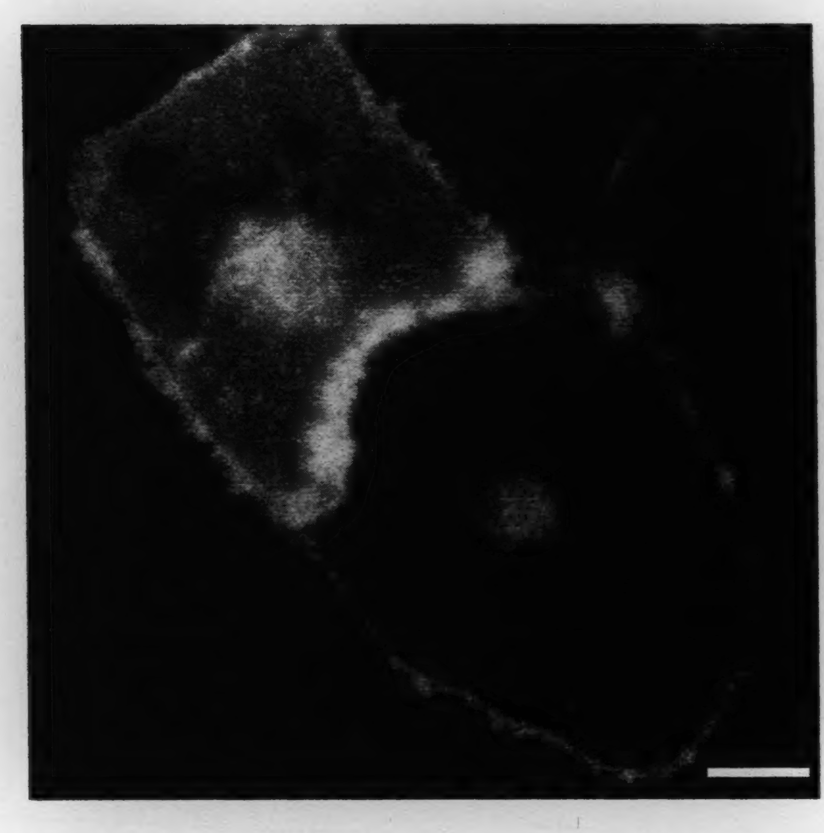
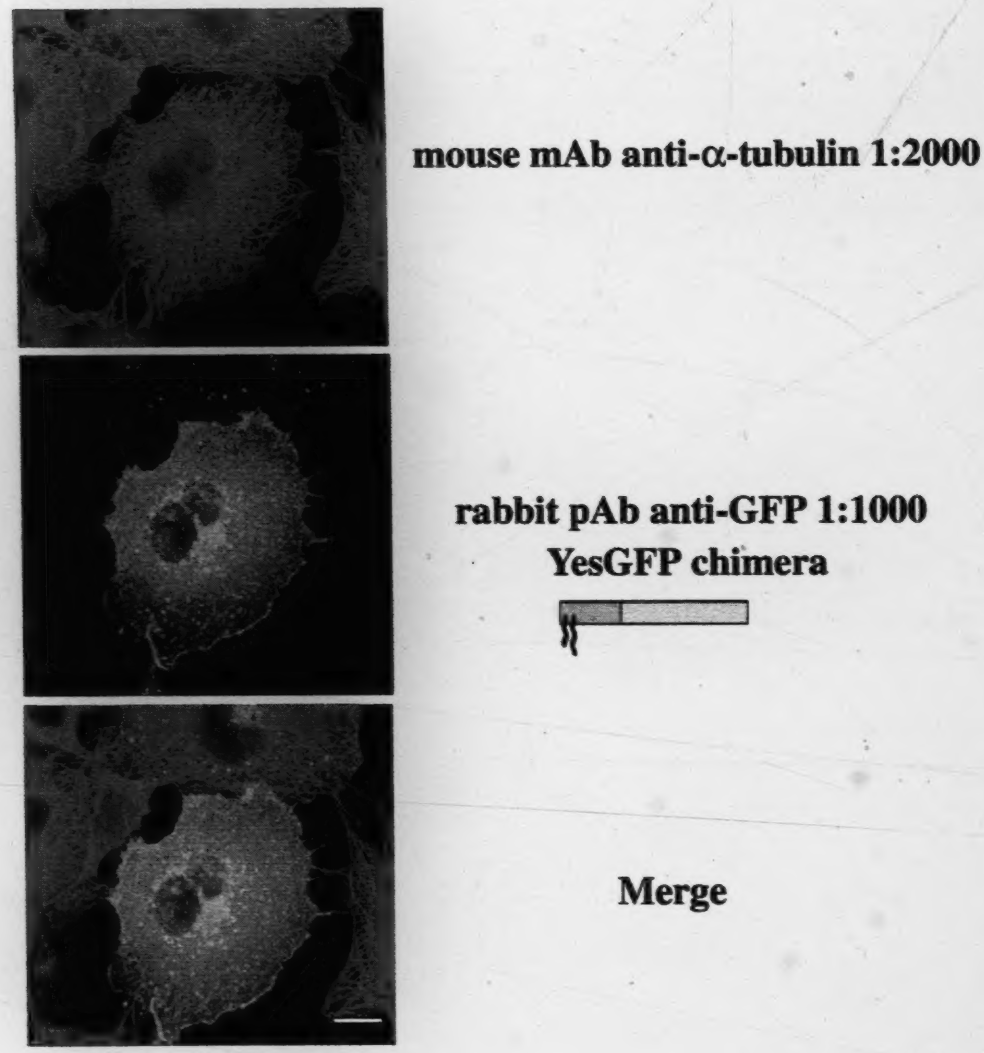


Figure 6.5. Enrichment of acylated GFP chimeras at cell-cell junctions. GFP fluorescence of live COS-7 cells expressing dually acylated YesGFP chimera. Intrinsic GFP fluorescence was detected by confocal laser scanning microscopy. Note enrichment of fluoresence at the edge of cells where their plasma membranes are in close approximation. Although both cells are expressing GFP at different levels, there is clearly a bright area of high GFP chimera density at the junction between them. Bar, 10 μm.



mouse mAb anti-α-tubulin 1:2000

rabbit pAb anti-GFP 1:1000
YesGFP chimera

Merge

Figure 6.6. Colocalization of endosomes containing acylated GFPs with microtubules in fixed COS-7 cells. The microtubule protein α-tubulin was detected with mouse anti-α-tubulin Ab followed by TR-conjugated anti-mouse Ab (top panel). The GFP chimera YesGFP was detected by incubation with rabbit anti-GFP Ab followed by FITC-conjugated anti-rabbit Ab (middle panel). Please note the alignment of endosomes on microtubule filaments. The merged image is shown (bottom panel). Bar, 10 μm.